BIOLOGICAL CONTROL OF ERWINIA AMYLOVORA BY ERWINIA HERBICOLA

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in Microbiology.

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ABSTRACT

A naturally-occurring *Erwinia herbicola* strain, Eh1087, inhibitory to *Erwinia amylovora* (Burr.) Winslow *et al.* (1920) was isolated from apple blossom (*Malus* X. *domestica* Borkh.) in a Canterbury orchard. Total populations of *E. herbicola* on apple blossoms in Canterbury and Hawkes Bay orchards were low (less than 50 cfu.blossom^{-1}) throughout flowering, increasing 100-fold at petal drop. *E. herbicola* populations at petal drop were more dominant in Canterbury orchards (30-50% of total) than in Hawkes Bay orchards (1-2% of total) in the seasons surveyed. *In vitro* inhibition of *E. amylovora* was observed for 20% of all bacterial strains isolated from Canterbury orchards and from none of the strains isolated from Hawkes Bay orchards under the conditions used.

Eh1087 established stable populations for at least 10 days post application when sprayed onto apple blossoms in the orchard. This strain produced a broad spectrum, non-peptide, β-lactam antibiotic *in vitro* that was bactericidal for *E. amylovora*. Eh1087 suppressed *E. amylovora* disease symptoms in immature pear fruit, excised apple shoots and in apple blossoms. The partially purified antibiotic of Eh1087 reduced the severity of symptom expression in immature pear fruit.

Tn*phoA*-insertion mutants of Eh1087 were created (Ant$^-$) that failed to inhibit *E. amylovora in vitro* and in immature pear fruit. Ant$^-$ mutants no longer produced an antibiotic inhibitory to *E. amylovora in vitro*. Tn*phoA* insertions in the Ant$^-$ mutants all mapped within a 1.5 kb region on a 200 kb indigenous plasmid of Eh1087.

A genomic library of Eh1087 was constructed and cosmids which complemented Ant$^-$ mutants to the Ant$^+$ phenotype *in vitro* and in immature pear fruit were selected.

A *Hin*dIII 8 kb cosmid fragment subclone, pAH8, weakly complemented Ant$^-$ mutant EhA17g. This complementation was enhanced by the presence *in trans* of an *Eco*R1 5 kb cosmid fragment subclone, pBE5B, which mapped approximately 6 kb away from the region of Tn*phoA* insertions.

Analysis of the proteins produced by these cosmid fragment subclones in *Escherichia coli* mini-cells revealed that pBE5B codes for three proteins, approximately 20, 34 and 41 kd in size and pAH8 codes for two proteins, approximately 28 and 39 kd in size. At least one of the proteins coded for by pAH8 must be essential to antibiotic synthesis in Eh1087. The possible relationship between the cosmid fragment-encoded proteins and b-lactam biosynthetic enzymes is discussed.

CHAPTER 1

INTRODUCTION

Fire blight is a disease of pipfruit and other rosaceous plants caused by the bacterium, *Erwinia amylovora* (Burr.) Winslow *et al.* (176). This disease has a world-wide distribution and results in serious economic losses to pipfruit industries in many fruit-growing countries (157). Although actual figures are difficult to determine, losses of up to 95% have been reported (156). In New Zealand crop damage is not significant overall, but the presence of fire blight impedes pipfruit export to countries which remain free of the disease, such as Australia and Japan.

Because of the devastation this disease can cause, fire blight is recognised as one of the most important bacterial plant diseases. However, despite the research effort plant pathologists have devoted to fire blight since the 1880's, much remains to be understood about the nature of this disease and the mode of action of *E. amylovora*.

Primary infection of blossoms and young shoots occurs during the spring. *E. amylovora* invades the plant via the blossom nectaries and other natural openings or wounds. Bacterial penetration of the underlying tissues causes necrosis and results in the typical blackened, shrivelled appearance of the blighted plant. As infection progresses, a characteristic bacterial ooze appears as an exudate from infected plant parts. This bacterial ooze is a source of inoculum for further spread of infection, mediated by insects, rain and man. The pathogen can overwinter in cankers on the tree, which may then provide a source for disease the following spring.

Because fire blight affects both blossoms and fruit spurs, crop losses are incurred in both the current season and the following season. Infected branches must be pruned out and in cases of severe infection, whole trees may be destroyed.

Current disease control strategies include winter applications of copper sprays and timed applications of the antibiotic streptomycin during blossom. Copper sprays are phytotoxic, causing russeting when sprayed during and immediately after blossom. Antibiotic applications are expensive and must be made prior to infection, creating the need for accurate disease forecasting. In addition, the use of antibiotic sprays can lead to the development of pathogen resistance and streptomycin-resistant *E. amylovora* has been isolated (36, 109, 111, 152).

The problems associated with the chemicals used to control fire blight prevent their registration in many countries and have prompted increased interest in the possibility of a biological control for fire blight.

Epiphytic bacteria antagonistic to *E. amylovora* have been isolated from host plants (47, 48, 58, 104, 119, 172). *Erwinia herbicola* (Lohnis) Dye (43), an organism often found closely associated with *E. amylovora*, has been the most frequently investigated potential biological control agent since discovery of its inhibitory activity in the 1940s (13, 14, 74, 119, 128, 129, 130, 154, 161, 171, 177, 178). A number of inhibitory strains of *E. herbicola* are currently undergoing limited orchard trials (76, 106, 146, 162).

There are many examples of the use of antagonistic bacteria in the control of plant pathogens, both fungal and bacterial (8, 70, 71, 92, 96, 113, 125, 169, 183), although the number of successful applications is small because of the complexity of the microbial interactions involved. To be effective, a biological control agent must successfully compete not only with the pathogen but also with the indigenous microbiota (124). Frequently, the efficacy of a biological control agent is subject

to large seasonal fluctuations, or varies according to geographical location or plant host variety (97). These variations are usually in response to unidentified environmental factors (97, 124).

Microbial competitive ability may be improved by the production of antibiotics inhibitory to the natural microbiota and secondary metabolites with antifungal or antibacterial activity are often implicated in disease control (170). The synthesis of these secondary metabolites is directly affected by the nutritional status of the producer organism (52, 164, 170).

The development of a successful biological control agent therefore relies upon a knowledge of the modes of action of both the control organism and the pathogen, as well as an understanding of the microbial ecology involved.

Numerous virulence factors for *E. amylovora* have been identified, including the extracellular polysaccharide amylovorin (6, 59, 139, 140, 160, 185), phytotoxins (34, 49, 60, 185) and siderophores (141, 159). These are recognised as factors contributing to the virulence of the pathogen, but are not essential for pathogenicity.

With the application of molecular biological techniques, significant advances have been made in the study of *E. amylovora* pathogenicity, which is now understood to involve multiple, clustered pathogenicity genes, all the products of which are not yet identified (10, 166, 168). The *hrp* (Hypersensitivity Response and Pathogenicity) gene cluster of *E. amylovora* has been identified and cloned (10) and the product of the *hrpN* gene, harpin, has been purified and identified as the elicitor protein of the hypersensitive response (HR) (168). The *hrp* gene cluster controls the HR in non-host plants and is assumed to also be involved in pathogenicity in host plants, as *hrp* genes are an absolute requirement for pathogenicity in many (but not all) bacterial plant pathogens that produce necrotic

disease (37). Non-pathogenic mutants of *E. amylovora* exist that are still able to produce the HR in non-host plants, indicating that *hrp* genes are not the sole pathogenicity determinant for *E. amylovora* and that disease specific (*dsp*) genes also exist that control disease development in the host plant. These *dsp* genes have not yet been identified, but mutants have been obtained (144, 160).

With a clearer understanding of *E. amylovora* pathogenicity at a molecular level, a successful disease control is more likely to be achieved, possibly by modification of the pathogen itself to create avirulent strains, incapable of producing disease, but which compete with the active pathogen at the infection site. In this way, Lindow (96) has demonstrated control of frost damage by *Pseudomonas syringae* with non-ice nucleating strains of the same species.

Molecuar biological techniques can also contribute to our understanding of the mode of action of various biological control agents. Biosynthetic pathways for antibacterial and antifungal secondary metabolites can be elucidated and the regulation of biosynthesis analysed. This knowledge then provides the potential for genetic manipulation of biological control agents to enhance their efficacy. For example, *Pseudomonas fluorescens* strains which produce antifungal antibiotics and siderophores suppress various fungal plant pathogens (8, 62, 70, 71, 102, 183). Improved protection from fungal disease has been observed by recombinant strains, modified to overproduce antifungal antibiotics (62, 102).

The use of genetically modified strains in the field remains a controversial issue, with the principal concern being the stability of the altered DNA in the environment (98). To effectively use genetically modified organisms in biological control it will be necessary to employ good ecological management practices in addition to the new technology.

This study examines the potential of a New Zealand isolate of *E. herbicola* as a biological control agent for fire blight and investigates both the ecology of *E. herbicola* in the orchard and the molecular mechanism of its inhibition of *E. amylovora*.

CHAPTER 2

BACTERIAL POPULATIONS IN NEW ZEALAND APPLE ORCHARDS

SUMMARY

Populations of total bacteria and *E. herbicola* were monitored on apple blossom in selected orchards at two different geographical locations in New Zealand (Canterbury and Hawkes Bay). In all four orchards surveyed *E. herbicola* populations remained negligible (less than 50 cfu.blossom^{-1}) throughout flowering, increasing rapidly at petal drop to reach levels of 1 x 10^3 cfu.blossom^{-1} (Hawkes Bay) to 1 x 10^5 cfu.blossom^{-1} (Canterbury). Total bacterial populations of 5 x 10^2 cfu.blossom^{-1} (Hawkes Bay) to 5 x 10^4 cfu.blossom^{-1} (Canterbury) were seen throughout flowering. Total bacterial populations increased 100-fold at petal drop in both locations. *Pseudomonas* spp. were predominant in Canterbury throughout all flowering stages and in Hawkes Bay after early flowering.

The possible role of *E. herbicola* and other epiphytic bacteria in biological control of fire blight is discussed in the context of the microbial ecology of the anthoplane.

INTRODUCTION

The fire blight pathogen, *E. amylovora*, was first reported in New Zealand at the beginning of this century (167). Since then, fire blight outbreaks have occurred sporadically, but the disease does not cause economically significant crop losses in this country.

A retrospective study in New Zealand (153) demonstrated that, using established prediction systems, the occurrence of disease outbreaks was less than expected. The low incidence of fire blight disease in New Zealand, despite conducive climatic conditions and high pathogen populations, suggests that *E. amylovora* could be subject to some form of natural biological control.

The possibility of biological control exerted by epiphytic bacteria is well recognised (33, 97, 124). Bacteria present on plant surfaces may reduce the disease potential of a pathogen through their interactions with the pathogen and the host plant, which influence the conditions of the infection court.

Surveys of epiphytic bacterial populations on blossoms in selected apple orchards were carried out in two consecutive years, with particular emphasis on *E. herbicola*, a known antagonist of *E. amylovora*.

METHODS AND MATERIALS

Orchard Surveys

In 1989 mature apple trees (*Malus* X. *domestica* Borkh. cv. Golden Delicious) were sampled in two orchards in Loburn, Canterbury. One orchard had a previous history of fire blight infection and the other no previous history of infection. In 1990 cv. Gala and cv. Royal Gala apple trees in Hawkes Bay were sampled from two orchards with no previous history of fire blight infection. Geographical locations of the orchards surveyed are shown in Figure 2.1. Streptomycin sprays, for the prevention of fire blight, were not used in the orchards surveyed. All orchards employed winter copper sprays and applied fungicide at 7-10 day intervals throughout blossom for the control of fungal diseases.

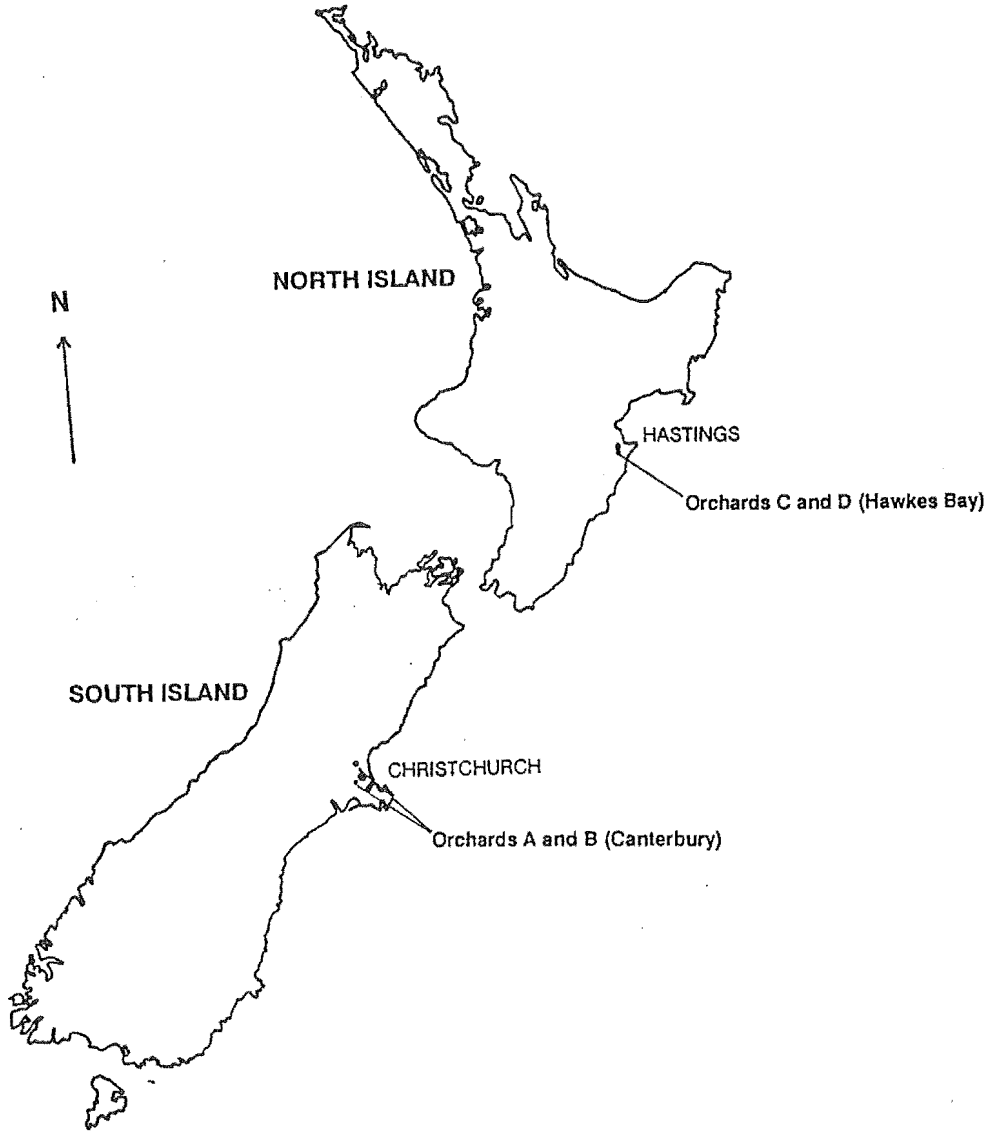


Figure 2.1: Geographical locations of orchards surveyed for bacterial populations in Canterbury (1989) and Hawkes Bay (1990).

Blossom Washes

Twenty blossom samples were collected daily at random from each orchard throughout the flowering period (Loburn, October 5 - November 11, 1989; Hawkes Bay, October 10 - November 8, 1990). Entire blossom clusters (5-7 flowers) were aseptically removed into a sterile plastic bag and stored on ice (2-4 hours) until further processed. Blossoms were washed for 2 minutes with vigorous shaking in 10 ml 0.85% sterile saline (w/v) + 1.5% peptone (w/v). Dilutions, 1:100 and 1:1,000 or 1:500 and 1:5,000, of the blossom wash were plated onto: a) Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bacto-agar) + cycloheximide at 50 $mg.l^{-1}$ (Sigma), to estimate total bacterial populations and b) modified Miller and Schroth agar (MS), lacking sodium taurocholate and thallium nitrate (109), to estimate populations of *Erwinia* spp. Plates were incubated at 30°C for 16-36 hours.

Bacterial Identification

For each sample date bacterial isolates were collected and partially identified using the rapid identification scheme outlined in Figure 2. 2. Methods used to identify bacteria were as follows:

Gram reaction

The Gram-stain reactions of bacteria were determined by mixing a loopful of cells taken from an LB agar plate (12-16 hour culture), in 3% KOH and observing viscosity changes, according to the method of Suslow *et al.* (147).

Fluorescence on Kings medium B

Bacterial cultures (12-16 hours) on Kings medium B plates (82) were observed for fluorescence under U.V. light.

Oxidation/fermentation test

To determine whether bacteria had oxidative or fermentative metabolism two tubes of base medium (72) containing 1% glucose (w/v) were stab inoculated and one tube

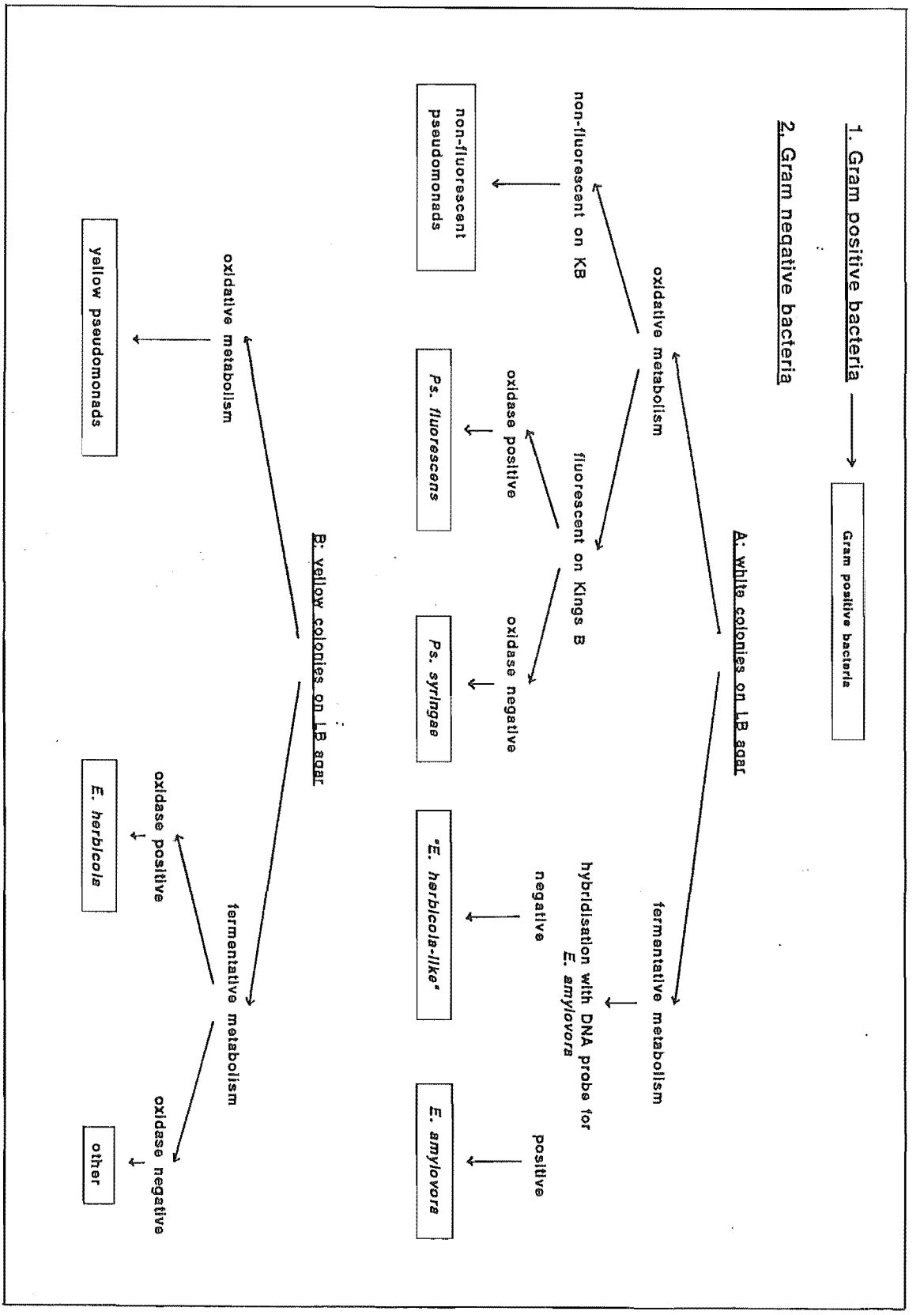


Figure 2.2: Rapid identification scheme used for preliminary identification of bacterial orchard isolates.

was sealed with 1-2 cm sterile paraffin liquid, according to the method of Lelliot and Stead (94). Tubes were incubated 3 days at 30°C. The production of a yellow colour (indicating acid production) in the unsealed tube but not the sealed tube indicated oxidative metabolism of glucose. Acid in both tubes indicated fermentative metabolism.

Oxidase test

This tests for the presence of cytochrome oxidase (85). A freshly prepared solution of 1% (w/v) tetra methylparaphenylene diamine hydrochloride (Sigma) in distilled water was pipetted over 24 hour bacterial cultures on LB agar plates. The development of a purple colour within 10 seconds was recorded as a positive reaction.

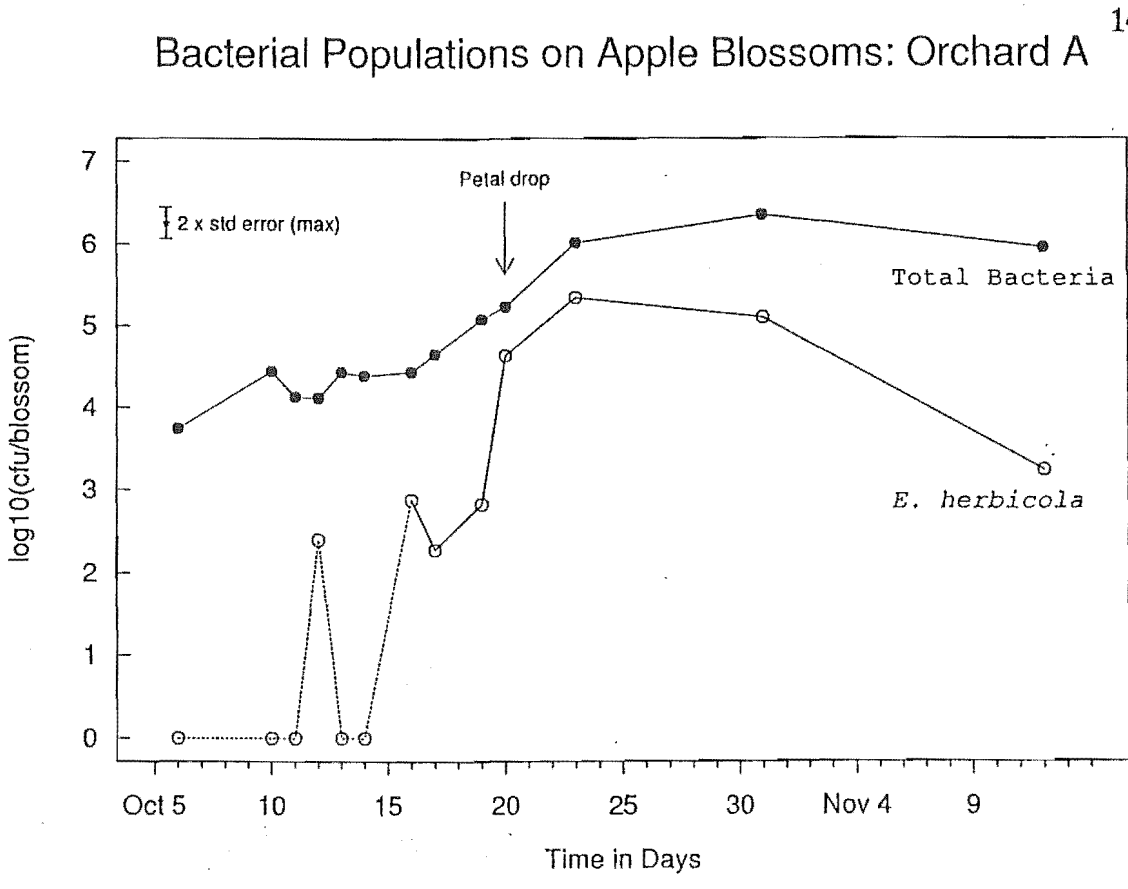
DNA hybridisation

To enable differentiation between *E. amylovora* and *E. herbicola*, yellow colonies growing on MS agar were blotted onto Hybond N+ nylon membranes (Amersham) and hybridised with a radio-labelled *E. amylovora* total genomic DNA probe (63). Colony blotting and hybridisation were carried out using methods outlined in Chapter 7.

RESULTS

Graphed bacterial population data from Canterbury orchards are shown in Figure 2.3 and from Hawkes Bay orchards in Figure 2.4. Certain similarities and differences were apparent in the bacterial populations of the orchards from different geographical locations.

In all four orchards surveyed population dynamics of total bacterial and *E. herbicola* populations throughout the blossom period were similar. Bacterial populations



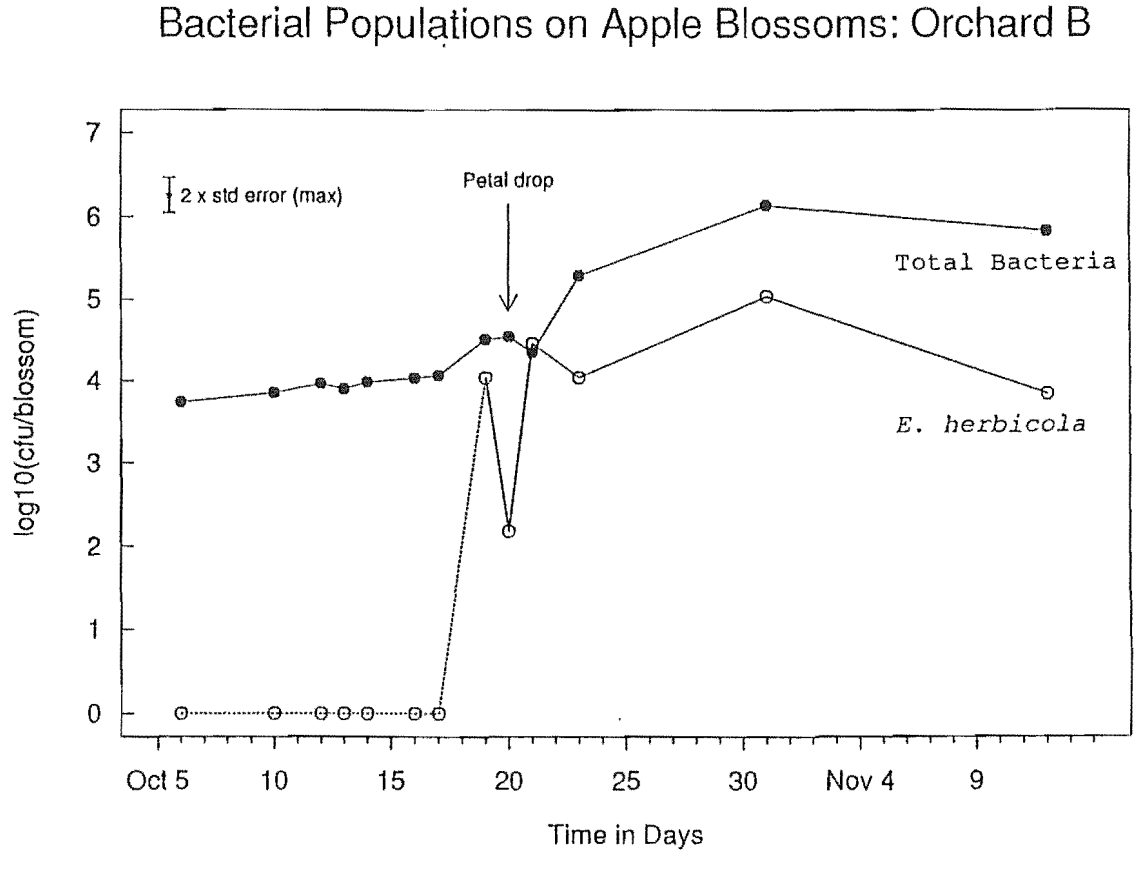
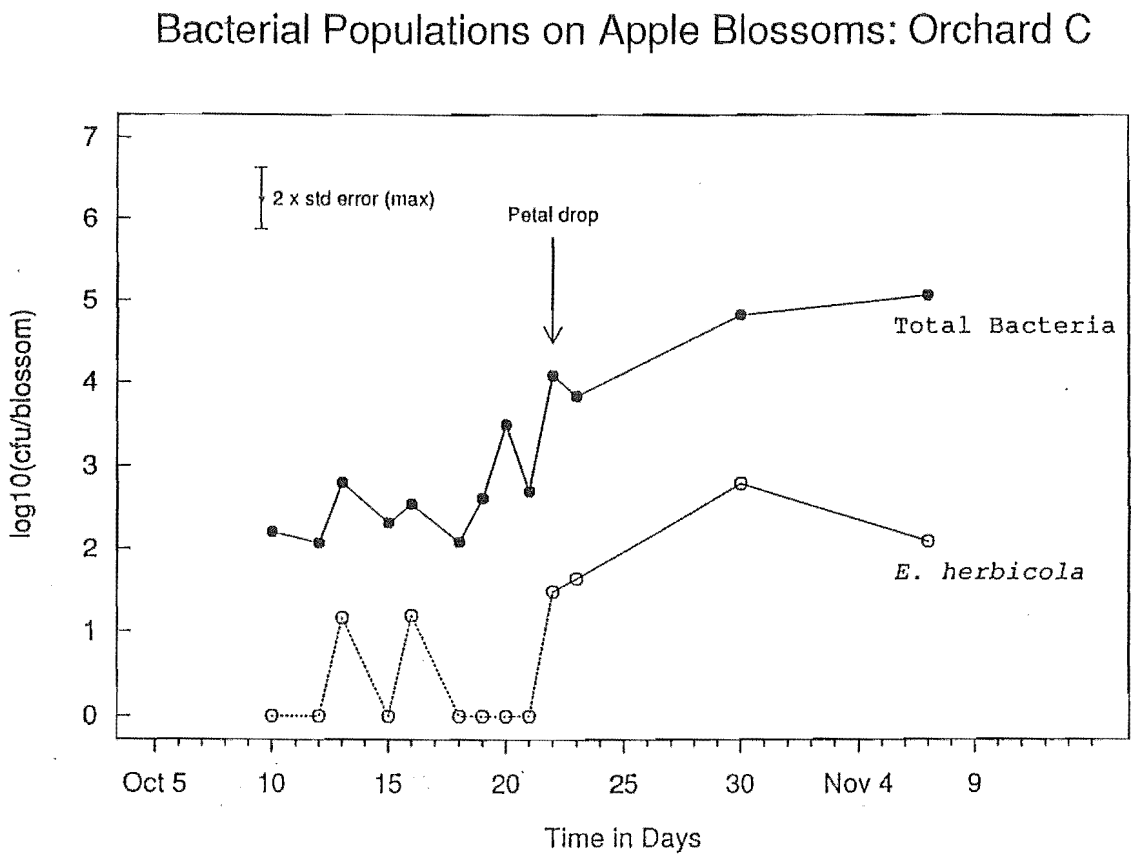


Figure 2.3: Populations of total bacteria and *Erwinia herbicola* on apple blossoms in Canterbury, orchards A and B (1989). *E. herbicola* means equal to zero are plotted at zero on the log scale (ie. approximately 1 cfu.blossom^{-1}). Time of 80% petal drop is indicated.



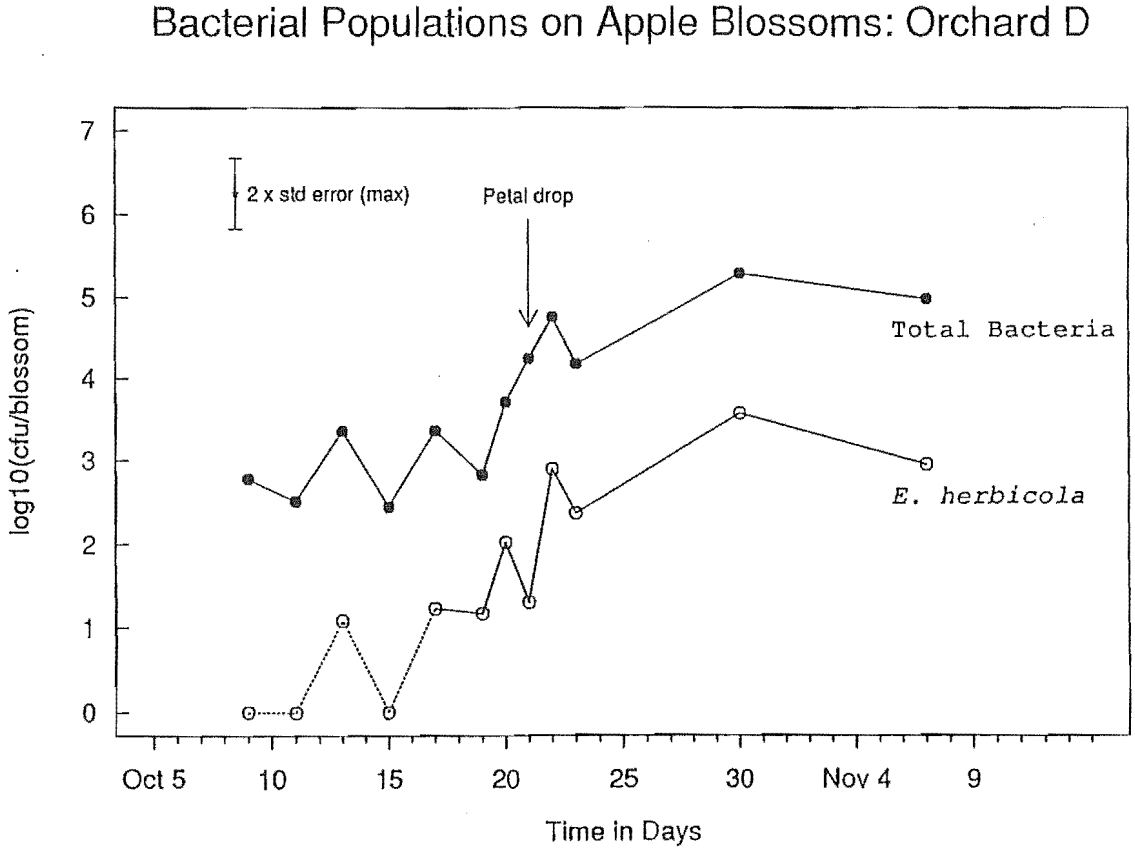


Figure 2.4: Populations of total bacteria and *Erwinia herbicola* on apple blossoms in Hawkes Bay, orchards C and D (1990). *E. herbicola* means equal to zero are plotted at zero on the log scale (ie. approximately 1 cfu.blossom^{-1}). Time of 80% petal drop is indicated.

remained stable throughout blossom, increasing at petal drop to reach maximum levels about ten days after the end of blossoming and then decreasing slightly as immature fruit develop. Total bacterial populations were higher in Canterbury orchards (1 - 5 x 10^4 cfu.blossom^{-1} during blossom to 1 x 10^6 cfu.blossom^{-1} at petal drop) than Hawkes Bay orchards (5 x 10^2 cfu.blossom^{-1} during blossom to 1 - 5 x 10^4 cfu.blossom^{-1} at petal drop).

In all orchards *E. herbicola* populations remained negligible (less than 50 cfu.blossom^{-1}) throughout blossom, increasing at petal drop to levels of 5 x 10^5 cfu.blossom^{-1} (Canterbury) and 5 x 10^2 - 1 x 10^3 cfu.blossom^{-1} (Hawkes Bay). At petal drop, *E. herbicola* populations represented 30 - 50% of the total bacterial population in Canterbury orchards, while in Hawkes Bay orchards *E. herbicola* represented only 1-2% of the total bacterial population.

In the Canterbury orchards the majority of the bacteria present were pseudomonads. *Pseudomonas syringae*, *P. fluorescens*, non-fluorescent and yellow pseudomonads were all isolated. White, "*E. herbicola*-like" isolates were uncommon. In contrast, bacterial populations in the Hawkes Bay orchards were more varied. Gram positive bacteria and "*E. herbicola*-like" bacteria were frequently isolated during early blossom, becoming less apparent as blossoming progressed and populations of *Pseudomonas* spp. and *E. herbicola* increased.

In both survey years weather conditions were not conducive to fire blight infection. (See Appendix B for weather data). No *E. amylovora* was isolated from any of the orchards and no fire blight symptoms were seen in any of the surveyed blocks, although fire blight was present in an adjacent pear block in one of the Canterbury orchards.

DISCUSSION

Blossom washing was chosen as the method of sampling epiphytic bacteria, being comparable with other studies carried out (99, 109, 119, 179). A comparison of methods for sampling epiphytic bacteria indicated that blossom washing was the most reliable method tested (42). Some degree of selectivity always exists with a sampling method that relies on bacterial culture. A nutritionally rich LB medium was chosen to determine total bacterial populations as it was likely to support growth of all organisms present. The efficiency of plating (E.O.P.) of the modified MS medium used was determined to be 70-100% relative to LB for selected strains of *E. amylovora* and *E. herbicola*. This was in agreement with the E.O.P. determined for the original medium (109).

In all four orchards surveyed some general population trends are shared: 1) total bacterial populations increase as flowering progresses to reach high numbers at petal drop (10^4-10^6 cfu.blossom^{-1}); 2) incidence of *E. herbicola* is low during flowering, increasing only at petal drop; 3) pseudomonads are predominant species throughout flowering. Because these trends prevail over different seasons and geographic locations and in different (related) apple varieties, it is reasonable to expect that they may be universal.

The only other study of epiphytic bacterial populations during blossoming (99) shows population dynamics similar to those in this study, most notably low *E. herbicola* populations and high populations of *Pseudomonas* spp. on pear blossoms. Overall bacterial population levels are in agreement with those reported previously (99, 153).

Monitoring *E. herbicola* populations was carried out in the context of an investigation for potential biological control agents. Low overall *E. herbicola* populations during blossoming suggest that the bacterium is a poor epiphyte on apple blossoms during the period that the trees are most susceptible to fire blight infection and, as such, may be a poor candidate for development as a biological control agent. Artificial

inoculation of host plants with *E. herbicola* (10^8 cfu.ml^{-1}, sprayed until runoff) has shown that the bacterium can colonise the blossom in the same way as does the pathogen (130, 181). However, orchard trials of *E. herbicola* control of *E. amylovora*, which have employed direct spray applications of viable bacteria onto trees, have reported variable protection (13, 14, 129, 154, 162). Survival of applied bacteria was not confirmed in these trials and the findings of this survey suggest that poor survival of applied *E. herbicola* may have contributed to the variable results obtained.

In all orchards surveyed, large pseudomonad populations were seen throughout blossoming. Given the high epiphytic capability of pseudomonads these bacteria may be more suitable as potential biological control agents for fire blight, provided strains antagonistic to *E. amylovora* can be found. (See Chapter 3 for details of screening orchard isolates for antagonistic activity to *E. amylovora*). *Pseudomonas* species inhibitory to *E. amylovora* have been investigated as potential control agents (96, 119, 172) and a *P. fluorescens* strain, A506, has been used effectively in control of fire blight on pear in the USA (174). It may be that use of compatible *E. herbicola* and *Pseudomonas* sp. could provide optimum control of disease (146).

Among the four orchards surveyed differences were evident with respect to the composition and size of microbial populations supported on apple blossoms. Hawkes Bay orchards contained smaller, more variable bacterial populations, with fewer pseudomonads, than did Canterbury orchards. Biological control trials on pear conducted in the USA indicated that *P. fluorescens* A506 more effectively colonised blossoms in cool, humid conditions, whereas *E. herbicola* C9-1 was a better coloniser in warm, dry seasons (R. McLaughlin, V. Stockwell pers. comm.). Lower pseudomonad populations in Hawkes Bay orchards may, in part, be explained by differences in climate, as the Hawkes Bay survey season (1990) was warmer and drier than the Canterbury survey season (1989).

"*E. herbicola*-like" organisms were more frequently isolated from Hawkes Bay orchards than from Canterbury orchards. These non-pigmented strains appear to predominate on plant surfaces in the United Kingdom (20) and studies of biological control by *E. herbicola* in the United Kingdom are mostly with these strains, rather than yellow pigmented *E. herbicola* (171).

These population differences highlight the influences such factors as climate and host plant variety can have on the microbial ecology involved and demonstrate the need to continue investigations relating to biological control over many seasons and locations. Large interseasonal population differences have been reported in both chemical (4, 68) and biological (76, 162) control studies. These may have arisen, at least partially, from differences in the microbial community at the time as environmental conditions and plant host species are known to influence populations of epiphytic bacteria on plants (93, 121).

Fungicide applications, for the control of fungal diseases, were made throughout blossoming at 7 day intervals in Hawkes Bay orchards and 10 day intervals in Canterbury orchards. In a study by Andrews and Kenerley (4) on the effect of a pesticide programme on non-target epiphytic bacterial populations of apple, metiram fungicide was shown to be toxic *in vitro* to epiphytic bacteria at dosage rates used for field applications. Bacterial populations in orchards where fungicide applications were made were reduced 10-1,000 fold, with fluorescent pseudomonads and lactic-acid bacteria being the most affected. In addition, copper sprays have also been found to reduce bacterial populations on cherry for a long period following spraying (21). The influence of such orchard management techniques on bacterial populations, and their implications for biological control, must be considered as well as the influence of natural environmental factors in any appraisal of potential biological control in New Zealand orchards.

CHAPTER 3

INVESTIGATIONS OF ORCHARD ISOLATES INHIBITORY TO Erwinia amylovora

SUMMARY

Orchard isolates from two different geographical locations were screened for inhibitory activity to *E. amylovora in vitro* and in plant bioassays. From one location (Canterbury) 20-30% of all isolates showed some inhibition *in vitro*. No isolates from the other location (Hawkes Bay) showed *in vitro* inhibition of *E. amylovora*. The relevance of screening procedures used is discussed.

Two strongly inhibitory isolates, *E. herbicola* Eh1087 and *P. fluorescens* Pf2075, were chosen for further investigation. Eh1087 produced a broad spectrum antibiotic that was bactericidal for *E. amylovora*. In contrast, the inhibitory activity of Pf2075 was bacteriostatic and specific for *E. amylovora*.

INTRODUCTION

Numerous species of epiphytic bacteria have been proposed as biological control agents for fire blight, including *E. herbicola* (13, 74, 162, 163, 171, 172, 177), *Pseudomonas* species (58, 106, 154, 155, 172, 174, 182), avirulent *E. amylovora* (58, 104, 107, 150) and *Arthrobacter* spp. (106).

In his review article, Leben (92) outlined the ways in which epiphytic bacteria can inhibit plant pathogens as: 1) antibiosis, 2) nutrient and/or site competition, 3) induction of a plant immune response and 4) alteration of plant physiology to

make it less conducive to the pathogen. All these mechanisms have been proposed to play a role in the inhibition of *E. amylovora*, although the relative importance of each is unknown.

Disease suppressing strains of *E. herbicola* are known to produce several antibiotics which are inhibitory to *E. amylovora in vitro* (13, 74, 163, 172, 177, 178). There are many examples of antibiotic producing bacteria giving biological control of plant pathogens (52, 170), although the actual importance of antibiosis in control by these agents is still unclear. There are conflicting reports of the correlation between *in vitro* antibiosis and biocontrol in the orchard by *E. herbicola* (9, 13, 177), but mutagenesis studies have clarified that antibiosis is important in disease suppression, in concert with other mechanisms, such as competition (14, 163, 177, 180). Antibiotic production is likely to confer a greater overall competitive ability onto producer strains.

E. herbicola and *E. amylovora* both multiply on the stigmas of blossoms of host plants (65, 173) and are therefore likely to compete for nutrients and/or colonisation sites. Nutrient competition by *E. herbicola*, enhanced by antibiosis, has been proposed as the mechanism by which *E. herbicola* excludes *E. amylovora* from the stigmas of hawthorn blossoms (173). *P. fluorescens* A506 suppresses fire blight in the orchard by pre-emptive exclusion of *E. amylovora* from infection sites on the plant (174) and has not been noted to produce any *in vitro* antibiotic active against *E. amylovora*.

In addition to competition, alteration of the host plant physiology may also contribute to disease control by *P. fluorescens* A506 as blossoms inoculated with this strain show reduced nectar secretion and pigmentation changes, the latter possibly indicating metabolism of plant phenolic compound(s), such as arbutin, to products toxic to *E. amylovora* (174).

Avirulent strains of *E. amylovora* have been noted to protect plants from subsequent infection with fire blight (57, 150). The induction of a host immune response by the avirulent strains has been suggested (57, 104) as a lag phase between inoculation and infection challenge is necessary for protection. Also, protection is non-specific, as bacteria pathogenic to other plants and non-epiphytic on host plants are able to protect (57). Furthermore, cell-free sonicates and DNA from avirulent *E. amylovora* protect plants from subsequent infection (104, 105).

Progress in examining the relative importance of different mechanisms of inhibition by bacteria has been made extremely difficult by the limitations of available methodology and a lack of basic knowledge about the organisation and function of the microbial communities involved. In this study, antibiosis was monitored as a screening mechanism to detect natural isolates inhibitory to *E. amylovora*.

METHODS AND MATERIALS

In vitro Inhibition Assay

Bacterial isolates from each sample date were tested for inhibition of *E. amylovora* on a minimal agar (69) supplemented with niacin (Sigma) at 50 $mg.l^{-1}$ (HSN). A highly virulent New Zealand *E. amylovora* isolate Ea8862 from the ICMP[1] was chosen as the indicator strain for all assays. HSN agar plates were prepared with soft agar overlay lawns of Ea8862 as follows: For each plate a 5 ml aliquot of soft HSN agar (0.8% agar) was melted and allowed to cool to 55°C, then inoculated with 100 μl of overnight (12-16 hr) LB broth culture of Ea8862 and poured over the surface of the plate. Single colonies of test bacteria were toothpicked onto the

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1 International Collection of Micro-organisms from Plants. Landcare/ Manaaki Whenua Research New Zealand Limited, Auckland, New Zealand.

prepared lawns. Plates were incubated at 30°C for 16-36 hours and antagonistic bacteria giving zones of inhibition of *E. amylovora* (see Figure 3.1) were recorded. Each isolate showing inhibition was retested at least twice.

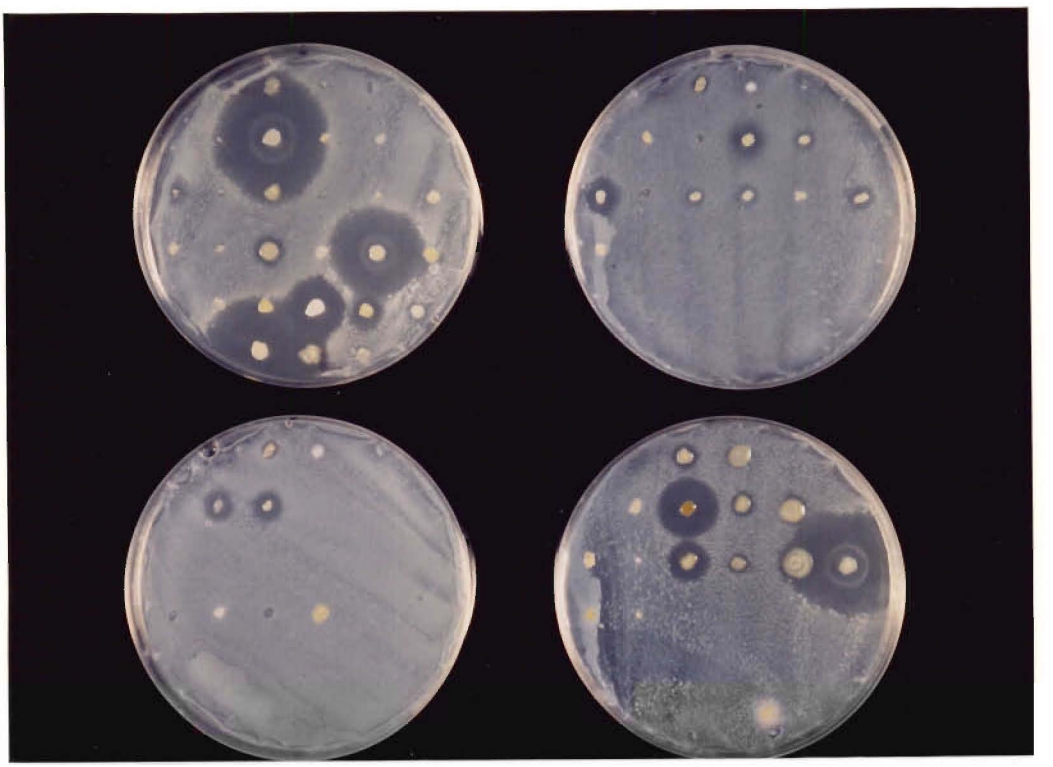


Figure 3.1: Screening of bacterial orchard isolates for inhibition of Ea8862 on HSN agar. Each isolate showing a zone of Ea8862 growth inhibition was re-tested at least twice.

Immature Pear Fruit Assay (IPFA)

Immature pears (*Pyrus communis* L. cv. Bartlett) were surface-sterilised for 10-15 minutes in 0.5% (w/v) sodium hypochlorite solution and then washed for 20 minutes in running water. Pears were aseptically sliced (3mm) and placed in sterile humidity chambers made from petri dishes containing water-saturated filter paper discs. Each treatment included 6-10 slices from at least three pears. Cells from overnight LB broth cultures of orchard isolates and *E. amylovora* Ea8862 were sedimented by centrifugation and resuspended in sterile saline 0.85% (w/v) to $O.D._{600}$ 0.2 (approximately 5×10^8 $cfu.ml^{-1}$). The centre of each pear slice was simultaneously inoculated with 50 μl of resuspended cells of Ea8862 and individual

isolates. Saline-only and pathogen-only controls were included in each assay. Pear slices were incubated at 22-26°C for 4-6 days and were scored positive for infection when water-soaking and/or ooze production was observed (See Figure 3.2).

Percent protection was measured on the first day showing 100% infection in the Ea8862 control as follows: %protection = (No. uninfected pear slices / Total no. pear slices) x 100.

Figure 3.2: Immature pear fruit assay, 3 days following inoculation of pear slices A). Pear slices inoculated with Ea8862 alone, B). Pear slices inoculated with Ea8862 and inhibitory *Erwinia herbicola* isolate Eh1087.

Apple Shoot Bioassay (APSA)

Freshly excised shoots (10 cm in length) from glasshouse grown apple plants (cv. Golden Delicious) were inoculated by injection through the stem mid-way between the apex and the first fully emerged leaf. Cell suspensions of the inhibitor and *E. amylovora* Ea8862 were used as for the IPFA. Stems were first inoculated with the inhibitor and then immediately re-inoculated at the wound site with Ea8862. A 25-

guage needle was used to deposit approximately 50 μl of cell suspension each time in a method similar to that of McIntyre *et al.* (105). Saline-only and pathogen-only controls were included in each assay. Apple shoots (10-12 per treatment) were maintained in water for 5-10 days at 22-25°C and scored positive for infection (Figure 3.3) when any of the following symptoms were seen: 1). stem necrosis extending from the wound site, 2). ooze production at/or extending from the wound site and 3). leaf wilt and necrosis. Percent protection was measured as for the IPFA.

Figure 3.3: Apple shoot bioassay, 5 days following inoculation A). Apple shoot inoculated with Ea8862 alone, B). Apple shoot inoculated with Ea8862 and inhibitory *Erwinia herbicola* isolate Eh1087.

Spectrum of Activity

Eh1087 and Pf2075 were tested for the inhibition of 35 different *E. amylovora* isolates obtained from the ICMP and of various identified orchard isolates (Table 3.1).

Table 3.1: Bacterial Isolates used to Assay Inhibition by Eh1087 and Pf2075

Isolate (ICMP No.)	Place of origin	Plant host
E. amylovora		
9149	New Zealand	*Pyrus pyrifolia*
9148	" "	" "
9146	" "	" "
1492	" "	*Pyrus communis*
1503	" "	" "
8863	" "	" "
8864	" "	" "
4450	" "	" "
1506	" "	*Crataegus monogyna*
1507	" "	" "
1493	" "	*Malus* X. *domestica*
1440	" "	" "
8862	" "	" "
8865	" "	" "
1540	United Kingdom	*Pyrus communis*
1536	" "	" "
1534	" "	*Crataegus monogyna*
1498	" "	*Malus* X. *domestica*
1537	" "	" "
1533	" "	*Cotoneaster* X. *crispii*
1532	" "	*Cotoneaster melanocarpus*
3860	USA	*Pyrus commununis*
3859	"	" "
4222	"	" "
1392	"	*Malus* X. *domestica*
1393	"	*Pyracantha* sp.
9109	Europe	*Crataegus monogyna*
9103	"	" "
9106	"	" "
9108	"	*Cotoneaster* sp.
9105	"	*Cotoneaster buxifolius*
9111	"	*Pyracantha* sp.
9110	"	*Mespilus germanica*
<u>New Zealand Orchard Isolates (not included in ICMP)</u>		
Pseudomonas sp.		
1027	Canterbury	*Malus* X. *domestica*
1085	"	" "
1070	"	" "
2063	"	" "
2054	"	" "
1070	"	" "
E. herbicola		
1082	"	" "
2043	"	" "
1037	"	" "
1101	"	" "
2076	"	" "
1077	"	" "
1030	"	" "
1004	"	" "
2071	"	" "

Overnight cultures of Eh1087 or Pf2075 were streaked onto HSN plates in a single band across the centre of each plate. Test *E. amylovora* isolates were cross-streaked perpendicular to the inhibitory isolate. Plates were incubated overnight at 30°C and zones of inhibition of cross-streaked bacteria were observed.

Rate of Growth

500 ml LB or HSN medium in side-arm flasks were inoculated with overnight cultures of Eh1087, Pf2075 or Ea8862 to give a nephelometer reading of 5.0% transmittance. Broth cultures were incubated at 30°C with vigorous aeration on a Gallenkamp orbital shaker (200 rpm) and % transmittance readings were taken hourly using a Unigalvo nephelometer.

Production of Inhibitory Activity in Liquid Culture

Overnight HSN broth cultures at 30°C were centrifuged to sediment cells. The broth supernatants were adjusted to pH 6.8 and sterile filtered. 20 µl drops of sterile broth supernatant were dropped onto an Ea8862 soft agar overlay lawn freshly prepared on HSN agar. Plates were incubated overnight at 30°C and zones of inhibition were observed.

During rate of growth experiments, samples were withdrawn at different stages of culture growth and tested as above.

Mode of Inhibition

To determine whether the inhibitory activity produced was bacteriostatic or bacteriocidal for *E. amylovora* HSN agar plates were prepared with spread lawns by dispersing 100 µl of overnight LB broth culture of Ea8862 over the entire surface of the plate with a sterile glass spreader. Single colonies of Eh1087 or Pf2075 were spotted onto the prepared plates and incubated overnight at 30°C. The next day inhibition zones were swabbed with sterile cotton buds which were then placed in sterile 3 ml LB broths and grown overnight at 30°C. The overnight

broths were streaked to single colonies on LB agar and MS agar and grown overnight. The presence or absence of colonies on the plates the next day suggested a bacteriostatic or bacteriocidal activity, respectively. To confirm *E. amylovora* identity colonies were blotted onto Hybond N+ membranes for hybridisation with an *E. amylovora* radio-labelled DNA probe (63) as previously described (Chapter 2).

RESULTS

Isolate screening

Bacterial isolates from Canterbury (total 180) and Hawkes Bay (total 108) were assayed for inhibition of *E. amylovora* Ea8862 on HSN agar. Of the Canterbury isolates, 20-30% showed some inhibition *in vitro*, while none of the Hawkes Bay isolates showed any inhibition. *Pseudomonas* isolates frequently inhibited *E. amylovora* very strongly *in vitro*, although this was inconsistent, and was not correlated with inhibition in plant bioassays. In fact, these inhibitory pseudomonads often were themselves pathogenic to pear and apple tissue.

The 10 bacterial isolates showing the strongest *in vitro* inhibition were also assayed using the IPFA and/or the APSA. Of these, one *E. herbicola* isolate (Eh1087) and one *P. fluorescens* isolate (Pf2075) showed inhibition of Ea8862 in plant bioassays. IPFA results for these isolates are shown in Figures 3.4 and 3.5, respectively.

Spectrum of Activity

Eh1087 inhibited all tested *E. amylovora* isolates. The isolates included both New Zealand and overseas strains of *E. amylovora* from a variety of hosts, including apple (*Malus* X. *domestica* Borkh.), pear (*Pyrus* spp.), hawthorn (*Crataegus monogyna* Jacq.), cotoneaster (*Cotoneaster* spp.) and pyracantha (*Pyracantha* spp.). An example of the inhibition observed is shown in Figure 3.6.

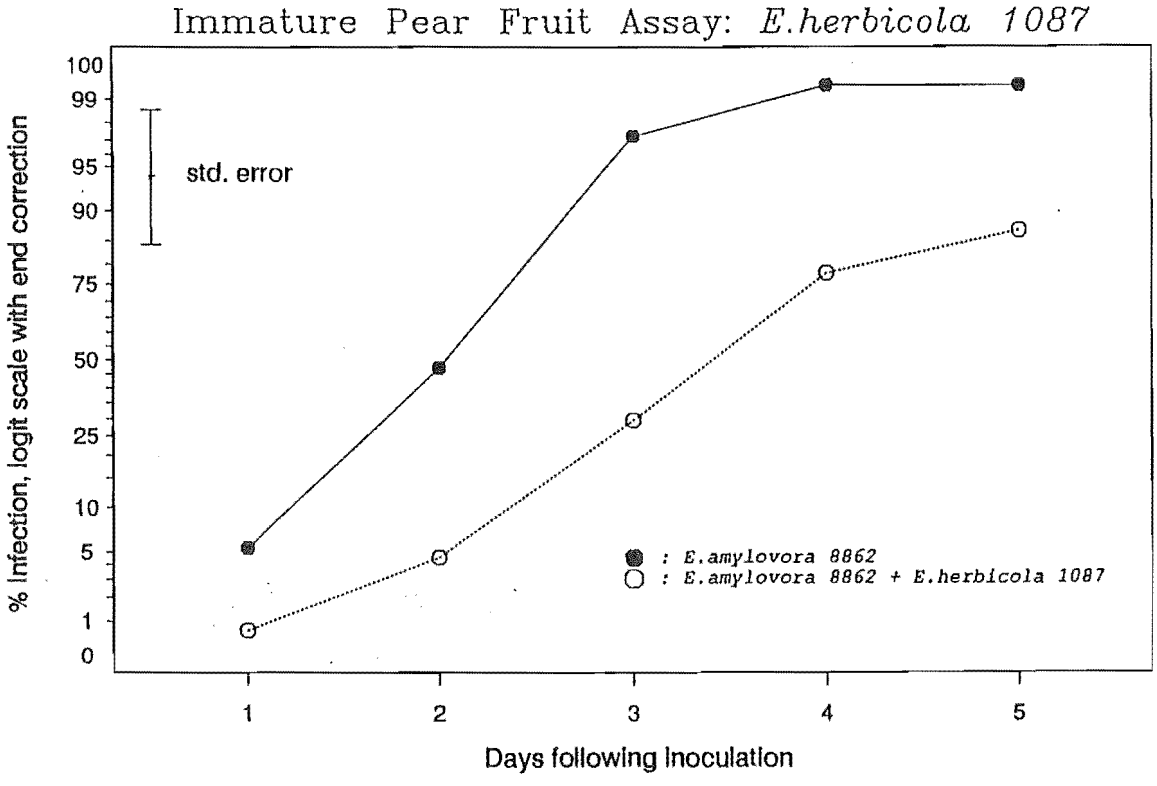


Figure 3.4: Immature pear fruit assay of disease suppression by Eh1087. Average values from 3 bioassays are shown. The standard error is indicated at the top left of the diagram.

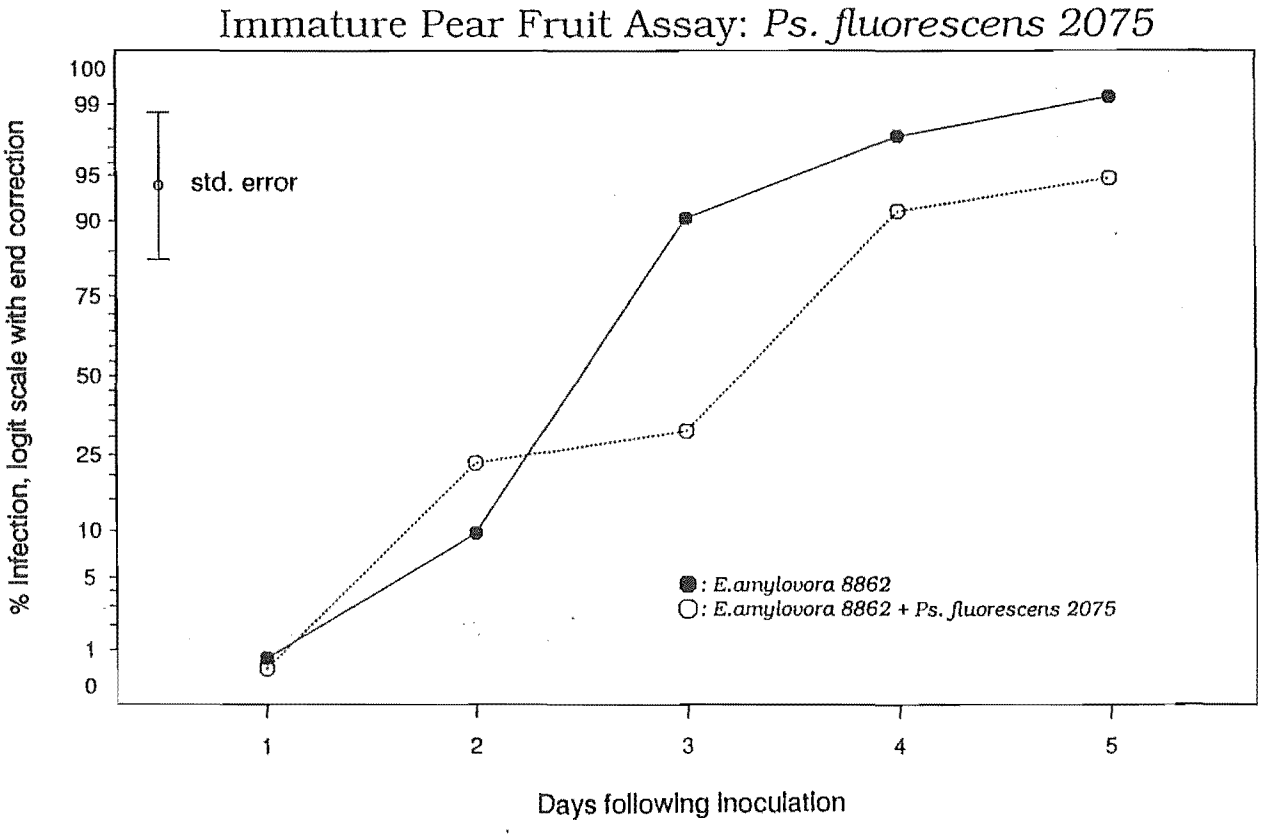


Figure 3.5: Immature pear fruit assay of disease suppression by Pf2075. Average values from 3 bioassays are shown. The standard error is indicated at the top left of the diagram.

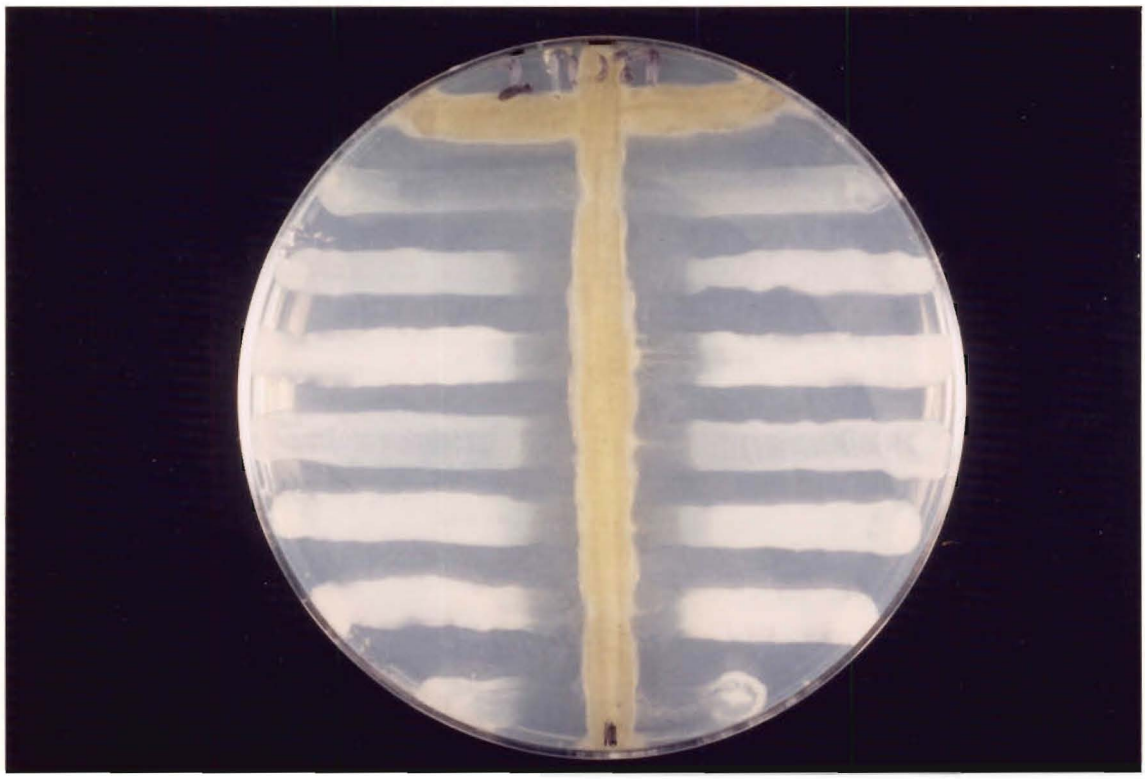


Figure 3.6: Inhibition of New Zealand *E. amylovora* strains by Eh1087 (vertical streak) on HSN agar. Cross-streaks from the top of the plate are Eh1087, Ea1440, Ea8863, Ea8864, Ea4450, Ea1503, Ea8862 and Ea8865.

Eh1087 weakly inhibited most of the *Pseudomonas* spp. (4/6) and *E. herbicola* (7/9) orchard isolates and did not self-inhibit. In contrast, Pf2075 inhibited all the *E. amylovora* isolates tested but failed to inhibit the *Pseudomonas* spp. and *E. herbicola* orchard isolates. No self inhibition was seen.

Rate of Growth

Rates of growth for Eh1087 and Pf2075 were compared with rate of growth for Ea8862 in LB broth and HSN broth at 30°C. Results are shown in Figures 3.7 and 3.8, respectively. In LB broth rates of growth of Eh1087 and Ea8862 were similar, while Pf2075 grew more slowly. In HSN broth, Ea8862 grew faster than either of the inhibitors.

Production of Inhibitory Activity in Liquid Culture

Cell-free broth supernatants from HSN broth cultures of Eh1087 demonstrated inhibitory activity against Ea8862. Timed sampling from Eh1087 broth culture revealed that the inhibitory activity is produced at the late log/early stationary phase. No inhibitory activity was observed in broth supernatants of Pf2075 cultures.

Mode of Inhibition

Viable *E. amylovora* could not be isolated from inhibition zones of Eh1087, indicating that the inhibitory activity of Eh1087 was bacteriocidal for Ea8862. In contrast, Ea8862 could be "rescued" from inhibition zones of Pf2075, indicating a bacteriostatic inhibition only.

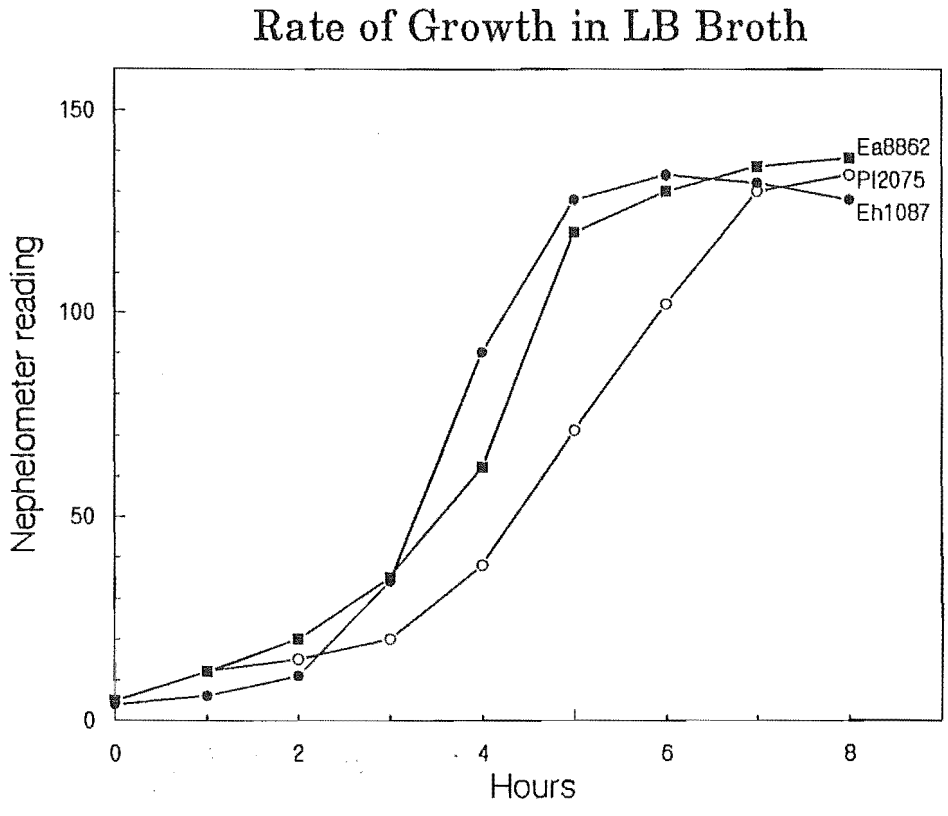


Figure 3.7: Rates of growth in LB broth culture of Ea8862, Eh1087 and Pf2075.

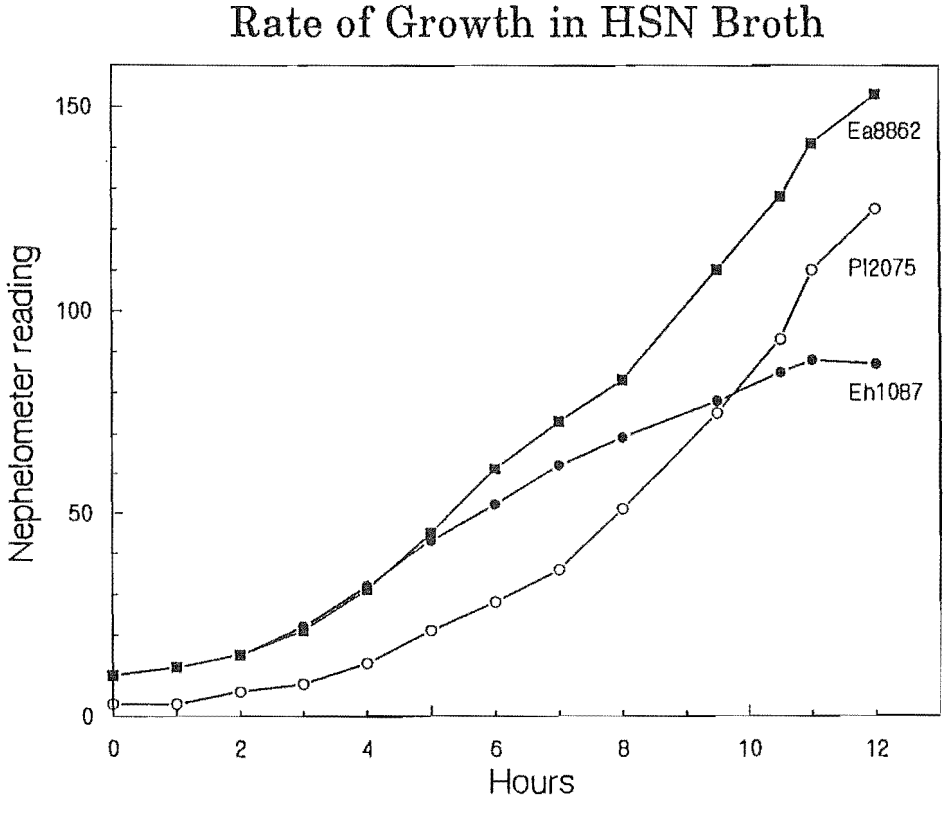


Figure 3.8: Rates of growth in HSN broth culture of Ea8862, Eh1087 and Pf2075.

DISCUSSION

Bacterial populations from apple orchards in different geographical locations differed from each other with respect to antibiotic/bacteriocin production *in vitro*. *In vitro* screening of orchard isolates by other researchers showed about 10% of all *E. herbicola* and "*E. herbicola*-like" strains tested produced *in vitro* inhibition (13, 16). This *in vitro* study similarly found that most *E. herbicola* strains tested were not inhibitory to *E. amylovora*. In contrast, Ishimaru *et al.* (74) report that *in vitro* antibiosis is commonly found in *E. herbicola* strains isolated from Michigan apple orchards.

HSN agar was chosen as the *in vitro* screening medium as it gave the largest number of positive results in preliminary screenings and larger inhibition zones produced by positive inhibitors than did other media tested, including a secondary metabolite medium (Appendix A), Kings medium B (82), LB medium, potato dextrose agar (Difco), phosphate-buffered potato dextrose agar, modified Burkholder medium (55, 118) and glucose-asparagine medium (180).

This method of *in vitro* screening for inhibitory bacteria selects for antibiotic and bacteriocin producers and may fail to detect bacteria which inhibit *E. amylovora* by other means. A lack of correlation between *in vitro* inhibition and disease suppression in the orchard has been observed (13). However, studies with non-producing mutants of antibiotic-producing strains of *E. herbicola* (13, 163, 177) suggest that antibiosis plays a major role in inhibition of *E. amylovora*, although microbial competition is also involved, as non-antibiotic-producing mutants of *E. herbicola* have a reduced, but not entirely ablated, ability to suppress disease.

Antibiotic and bacteriocin production by plant-associated bacteria *in vitro* has been shown to be highly influenced by the media used (this study, 2, 61, 112, 165, 178, 180), so the absence of antibiotic/bacteriocin production *in vitro* cannot absolutely

indicate that antibiotic production does not occur *in planta*. A highly significant correlation between *in vitro* inhibition and *in vivo* disease suppression has been observed for *E. herbicola* and fluorescent pseudomonad strains inhibitory to fungal and bacterial phytopathogens (81, 183). Microbial competition also contributed to the biological control effected by these strains. The correlation seen between *in vitro* and *in vivo* pathogen suppression must reflect use of an appropriate *in vitro* screening medium.

Two of the strong *in vitro* inhibitors of *E. amylovora* also inhibited in the IPFA and APSA. Nicholson *et al.* (119) found that approximately 25% of *E. herbicola* and fluorescent strains of *Pseudomonas* spp. tested were inhibitory to *E. amylovora* in immature pear fruit. Although the sample size was small in this study (total 10) it was also found that 20% of strains tested inhibited *E. amylovora* in plant bioassays.

The IPFA was developed to use as a convenient laboratory-based assay to identify strains of bacteria that are likely to suppress fire blight infection in the orchard (11, 13). The IPFA does not always correlate well with protection on mature trees and is unable to distinguish between antagonists of medium to high efficiency (13, 171). Because of this, scoring of bioassays in this study did not attempt to record differences in severity of symptom expression but only the presence or absence of disease symptoms themselves. This scoring system gives a more stringent measure of protection than one in which a graduated response is recorded.

A non-specific host response was observed when pathogen challenge was delayed 24 hours after inoculation of the pear slice with the inhibitory isolate. Heat-killed Ea8862 gave some protection from disease when inoculated 24 hours prior to challenge with virulent Ea8862 (Results not shown). Pre-inoculation with avirulent or heat-killed *E. amylovora* or with cell-free sonicates of the pathogen have also been shown to give some protection from fire blight disease in plant bioassays (104, 105, 182). In this study there was no apparent protection by heat-

killed Ea8862 when inoculated simultaneously with the virulent pathogen. All inoculations of test bacteria and pathogen were therefore carried out simultaneously.

Other investigators generally employ a delay of 3-24 hours between inoculation and pathogen challenge in plant bioassays (11, 35, 58, 66, 171) and in mature trees (14, 128). These workers have commented that the protective effect exerted depends upon the delay time before pathogen challenge (57, 66). *P. fluorescens* A506 effectively suppresses fire blight in the orchard by competitive exclusion when inoculated 72 hours in advance of the pathogen (174), but fails to suppress *E. amylovora* on pear blossom when co-inoculated with the pathogen. Taken together, these findings suggest that co-inoculation in plant bioassays may yield false negative results, but is less likely to yield false positive results than when delayed challenge is used. Because of the inability to perform orchard trials with virulent *E. amylovora* in New Zealand, it was considered more appropriate to employ a screening procedure which carries more risk of false negative results than of false positive results.

The inhibitory behaviour of isolates Eh1087 and Pf2075 was compared with respect to spectrum of activity and mode of action. Pf2075 and Eh1087 inhibited all *E. amylovora* strains tested *in vitro*. The fact that New Zealand inhibitory isolates are equally inhibitory to local and overseas *E. amylovora* strains indicates that the proposed biological control situation existing in New Zealand is not due to some unique character of local *E. amylovora* strains. (All New Zealand and overseas *E. amylovora* strains tested have appeared identical in biochemical tests and in DNA hybridisation studies carried out in this lab, C. N. Hale and R. Clark, pers. comm.).

Eh1087 produced a broad-spectrum antibacterial effect that was bacteriocidal for *E. amylovora* and also weakly inhibitory towards orchard isolates of *E. herbicola* and *Pseudomonas* spp. In contrast, the inhibitory activity of Pf2075 was bacteriostatic and specific for *E. amylovora*.

The antibacterial activity of Eh1087 could be detected in the supernatant of late log/early stationary phase broth cultures in HSN broth. No inhibitory activity was observed in HSN broth culture supernatants of Pf2075 under the experimental conditions tested, which included incubation at 25° and 30°C, with and without vigorous aeration.

Rate of growth studies were carried out to determine relative rates of growth of *E. amylovora* and the inhibitory isolates in HSN medium. Riggle and Klos (129) found that on media containing sugar concentrations comparable to pear nectar *E. herbicola* consumed all the organic nitrogen in the medium and suggested that bacterial competition for nutrients in the medium could cause depletion of *E. amylovora*. Hattingh *et al.* (66) suggested that *E. herbicola* inhibition of *E. amylovora* could be caused by depletion of organic nitrogen in plants by *E. herbicola*, which is able to utilise inorganic nitrogen. However, they were unable to demonstrate competition for any single amino acid in apple blossoms and IPFA results were inconclusive. Wodzinski *et al.* (181) observed that three differently inhibiting strains of *E. herbicola* grew at the same rate *in planta*, suggesting that depletion of nitrogen sources was not the mechanism of inhibition of *E. amylovora*.

Although the "N-depletion" hypothesis has lost favour among researchers in this field, it was felt necessary to determine that growth rates of pathogen and inhibitor were not markedly different in the *in vitro* assay medium used in this study. Accepting that behaviour of bacteria in broth culture can differ from that on solid media, it was found that *E. amylovora* Ea8862 grew at a faster rate in HSN than

did either of the inhibitory isolates tested. This finding suggests that a nutrient competition is unlikely to be responsible for the inhibition observed *in vitro*. The relative growth rates observed and the presence of inhibitory activity in cell-free culture supernatants of Eh1087 indicates production of an antibiotic by this strain when it is grown under limiting conditions.

Acidification of the environment, concommitant with nitrogen depletion, has also been observed to cause inhibition of *E. amylovora in vitro* (15, 129). In HSN broth culture Eh1087 acidification of the medium causes a pH drop from 6.8 to 4.0-5.0. Sterile culture supernatants, readjusted to pH 6.8, were still inhibitory to *E. amylovora*. The pH of inhibition zones present on solid HSN agar were measured with a surface pH electrode and were found to be pH 5.5 or higher. This pH was not inhibitory to Ea8862 in itself. (Results not shown).

The inhibitory activity of Pf2075 was lost on lyophilised storage and in frozen glycerol culture. Loss of bacterial viability or inhibitory activity under longterm storage conditions has been reported before (91, 118). Because of the loss of inhibitory activity and because Pf2075 proved to not be amenable to transposon mutagenesis, investigations on this strain were terminated.

CHAPTER 4

SURVIVAL OF APPLIED Erwinia herbicola IN ORCHARD AND GLASSHOUSE TRIALS

SUMMARY

Survival of applied *E. herbicola* Eh1087 was investigated on apple blossoms in the orchard and the glasshouse at three different application dates throughout flowering. Bioassays of *E. herbicola* control of fire blight disease on excised apple blossoms maintained in humidity chambers were conducted in conjunction with survival experiments.

Applied Eh1087 on apple blossoms in the orchard (application rate 7×10^7 cfu.blossom^{-1}) showed only a 10-40% survival rate at four days post application. However, applied *E. herbicola* populations still remained 400- to 800-fold higher than naturally epiphytic *E. herbicola* populations for at least ten days post application. In glasshouse conditions applied Eh1087 populations reached levels 10- to 30-fold higher than naturally epiphytic *E. herbicola* populations. Most efficient colonisation by Eh1087 was seen at mid-flowering application.

At early and mid-blossom stages applied Eh1087 gave 70% to 80% protection from fire blight disease, while late blossom applications gave only a 36% protection from disease.

INTRODUCTION

Antibiosis by *E. herbicola* has been determined to play a major role in biological control of *E. amylovora*, although microbial competition is also involved (163, 177). In order for biological control to be effective, the introduced *E. herbicola* strain must establish a stable population by successfully competing with the indigenous microflora, including the pathogen.

Surveys of naturally occurring *E. herbicola* on apple and pear blossom have indicated that epiphytic *E. herbicola* populations are low (77, 99), suggesting that *E. herbicola* may be a poor epiphyte. Orchard trials of different strains of *E. herbicola* have given variable control results but have not investigated survival of applied bacteria (13, 14, 162).

In this study the survival ability and biological control ability of a spontaneous rifampicin-resistant mutant of Eh1087 was investigated on apple blossoms in the glasshouse and compared with survival on apple blossoms in the orchard. Natural *E. herbicola* populations on blossoms were also monitored.

METHODS AND MATERIALS

Orchard Survival of Applied Eh1087

An overnight culture (12-16 hours) of Eh1087 (Rif^R) was grown in Luria-Bertani (LB) broth + rifampicin (Sigma, 50 $\mu g.ml^{-1}$) at 30° C with aeration to $O.D._{600}$ 1.0. Cells were sedimented by centrifugation (10 min, 6,000 rpm, Sorvall GSA) and resuspended in sterile saline to $O.D._{600}$ 0.9.

Individual apple trees (cv. Golden Delicious) were sprayed until run-off with the bacterial suspension using a hand-held sprayer at three different application dates throughout flowering (2 October, 7 October, 13 October 1991). Control

applications of saline only were also made at each application date. Bacterial counts were taken at one hour, two days, four days and ten days post application as follows: 10 samples of 5 blossoms each were collected aseptically and washed in 10 ml 0.85% sterile saline (w/v) + 1.5% peptone (w/v). Serial ten-fold dilutions (1:50 - 1:50,000) of the blossom wash were plated onto modified MS medium supplemented with rifampicin to determine populations of surviving Eh1087 on blossoms. Blossom washes from the saline control treatments were plated onto modified MS (to determine total epiphytic *Erwinia* population) and modified MS + Rif (to determine level of Rif^R *E. herbicola* in natural epiphytic populations).

For all treatments colonies from one plate for each sample were blotted onto Hybond N+ membranes (Amersham) and hybridised to the ^{32}P-labelled total DNA *E. amylovora* probe according to the method of Hale and Clark (63) to differentiate between *E. amylovora* and *E. herbicola*.

Glasshouse Survival of Eh1087

Blossom-bearing branches were removed from apple trees at each of the three application dates throughout flowering. Branches were recut under water and maintained in glasshouse humidity chambers (95% r.h., 20°C) in 0.2% (w/v) Benlate™ (Du Pont) solution in distilled water. Forty blossom clusters were used for each treatment. Eh1087 was applied and sampled as for orchard trials.

Biological Control of Ea8862 by Eh1087

Blossom-bearing branches were removed from the orchard and maintained in humidity chambers. Blossoms (100 per treatment) were sprayed with a bacterial suspension of Eh1087, as in survival experiments. A bacterial suspension of *E. amylovora* Ea8862, prepared in the same way as the Eh1087 suspension, was applied one hour later. Blossoms were left 24 hrs at 100% r.h., 16°C and then transferred to 95% r.h., 20°C and monitored daily for symptoms of infection.

RESULTS

Applications of Eh1087 onto apple blossom in the orchard show a 100- to 1,000-fold decrease in surviving bacteria for 4 days post application. Established populations after 10 days are 400- to 800-fold higher than naturally present *E. herbicola* levels, with the exception of late blossom applications which fail to establish at levels higher than natural populations (Figure 4.1).

Eh1087 applied onto blossoms in the glasshouse (Figure 4.2) rapidly establish populations of 10^7 - 10^8 cfu.blossom^{-1}. The naturally epiphytic *E. herbicola* population also increases under these growth conditions to levels of 10^6 - 10^7 cfu.blossom^{-1}.

Bioassays of fire blight disease control by Eh1087 on blossoms in the glasshouse are shown in Figure 4.3. At all blossom stages disease control at day 7 was significant by chi square analysis ($p > 0.05$). Most effective disease control was seen at mid-blossom.

DISCUSSION

Orchard applications of Eh1087 at different stages throughout flowering show that artificial application can result in established populations of the strain at 10 days post application, although up to 90% loss of viability in the applied bacteria occurs. It is interesting to compare these results with survival trials carried out in Poland with *E. herbicola* strain Eh112Y (179), where bacterial populations 10 days post application were restored to original inoculum levels.

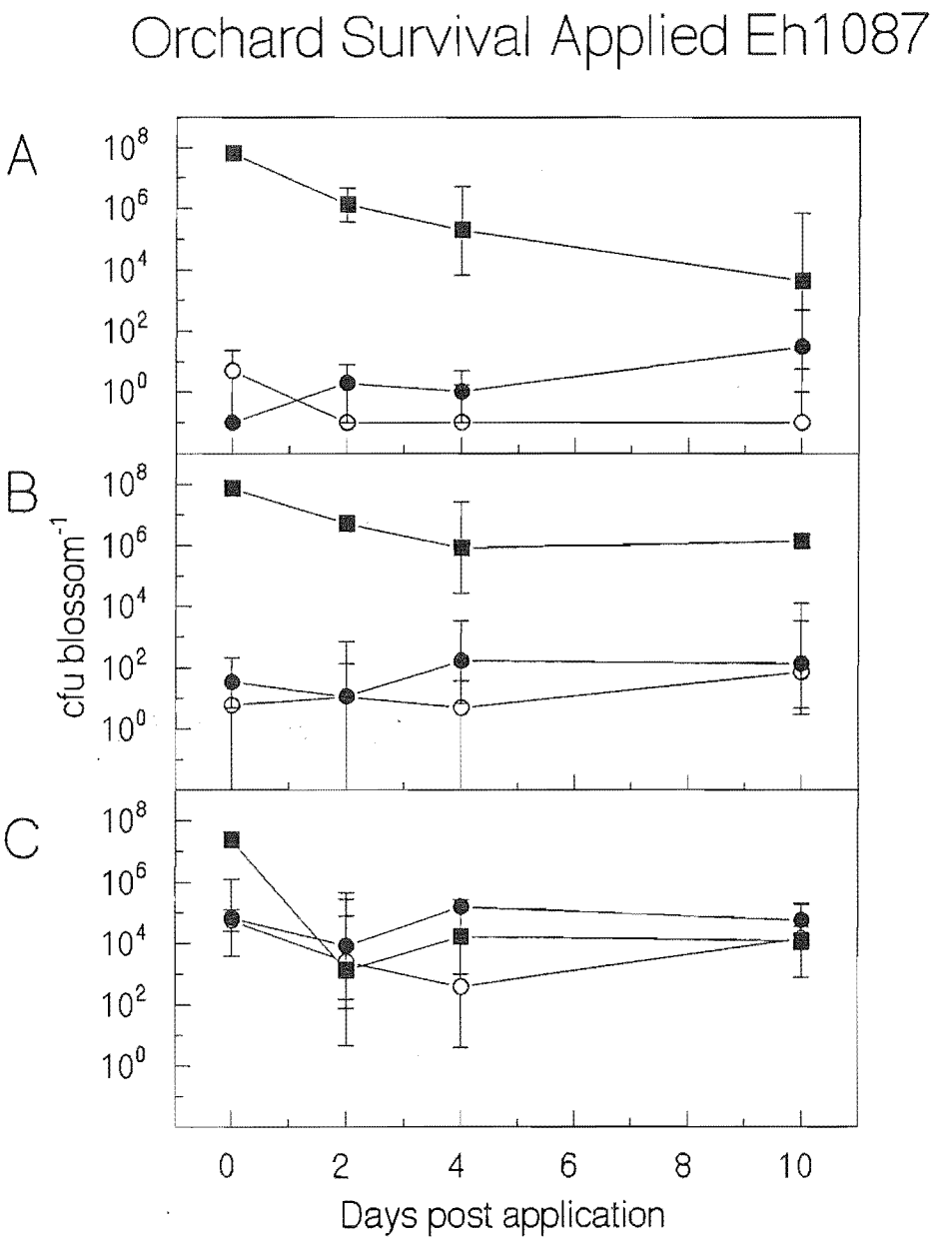


Figure 4.1: Survival of Eh1087 in the orchard when applied at A). Early blossom, B). Mid blossom and C). Late blossom. 95% confidence intervals are shown.

■—■ Eh1087

●—● epiphytic *E. herbicola*

○—○ Rif^R epiphytic *E. herbicola*

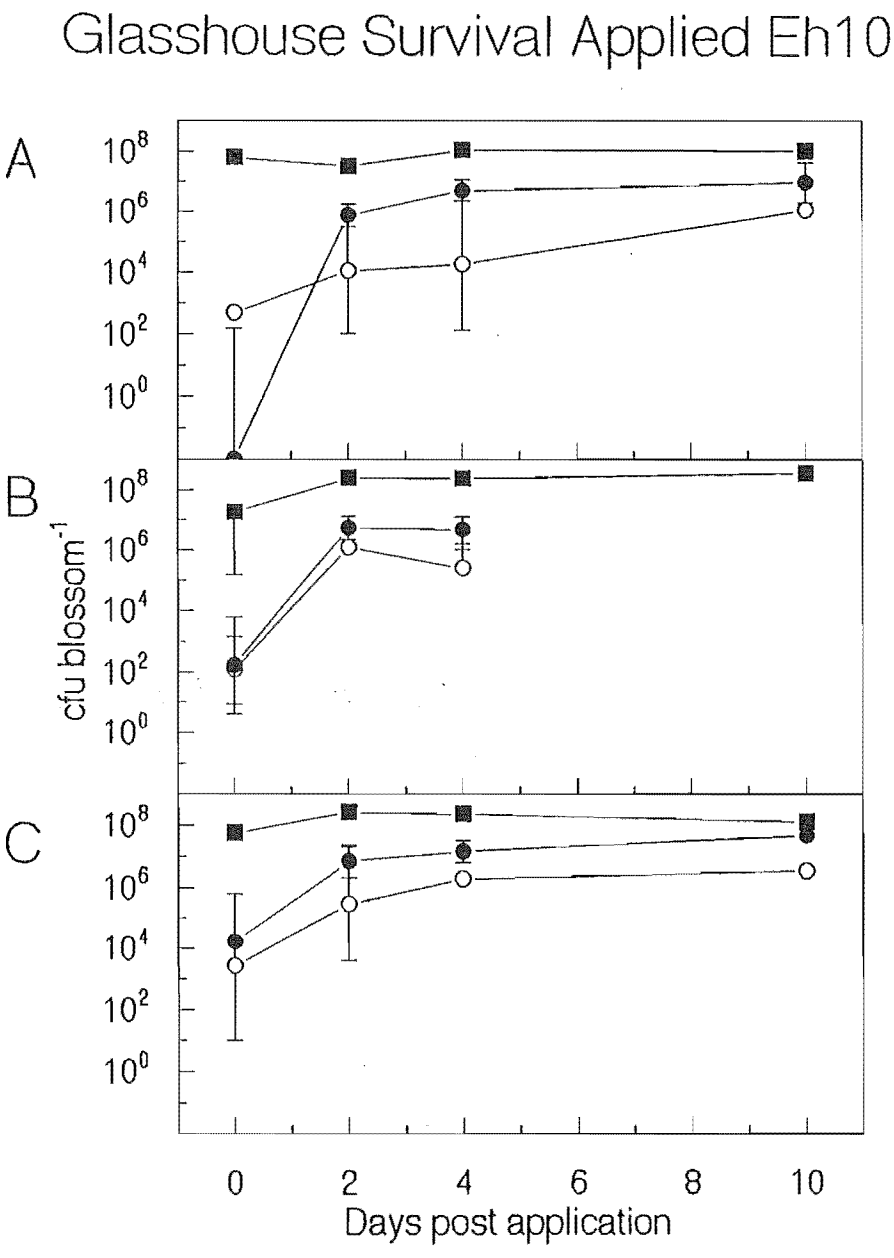


Figure 4.2: Survival of Eh1087 in the glasshouse when applied at A). Early blossom, B). Mid blossom and C). Late blossom. 95% confidence intervals are shown.

■—■ Eh1087

●—● epiphytic *E. herbicola*

o—o Rif^R epiphytic *E. herbicola*

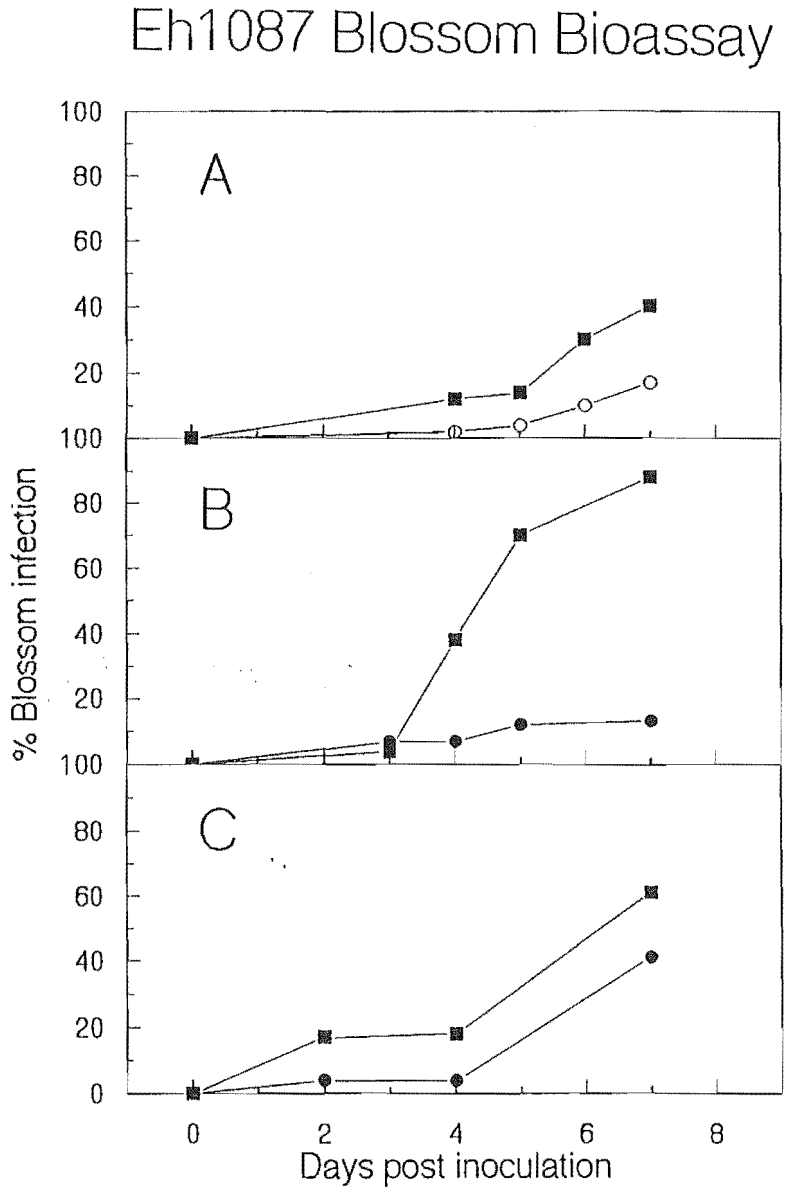


Figure 4.3: Blossom bioassay of Eh1087 control of Ea8862 in the glasshouse at A). Early blossom, B). Mid blossom and C). Late blossom. Disease control by Eh1087 was significant ($p > 0.05$) at all blossom stages.

■—■ Ea8862

●—● Ea8862 + Eh1087

At late flowering populations of Eh1087 did not establish at levels higher than did the natural populations. This may have been due to poor competition with epiphytic bacteria, as levels of epiphytic bacterial populations were higher at late blossom (4×10^5 cfu.blossom^{-1}) than at early and mid-blossom stages (2×10^2 - 2×10^3 cfu.blossom^{-1}). Alternatively, rain at the time of application (8 mm on day of inoculation and 2 mm on day 1) may have washed the applied bacteria off the stigmas, as is seen to happen with epiphytic *E. amylovora* (151).

Glasshouse conditions of optimal temperature and humidity enabled apple blossoms to support high bacterial populations. At all blossom stages, applied Eh1087 established readily, without large losses in viability.

In orchard applications of Eh1087 sample variance tends to increase with time. This could reflect changes in the spatial distribution of the applied bacteria, as a uniform distribution immediately after application becomes more uneven with time as bacteria respond to microclimates and microniches which exist on different areas of the plant. Similar observations have been made for applications of *E. herbicola* onto beans (42). This trend is not seen with glasshouse applications of Eh1087 where limiting conditions are less likely to occur. This is also reflected in sample variance for glasshouse populations of naturally epiphytic bacteria, which decrease with time in the glasshouse, possibly indicating spread and colonisation of all niches made available in the absence of limiting climatic factors.

Previous investigations have shown that the level of fire blight control by epiphytic bacteria is dependent upon their population size (12, 154). However, in this study, there was no correlation between biological control and population levels of Eh1087 in the glasshouse. The best disease control was seen at mid-blossom, although population levels of applied Eh1087 were the same at all blossom stages. This indicates that Eh1087 survival ability is not the only factor influencing the efficiency of disease control. This was also found to be the case for control of frost

injury by *P. syringae* (95). Different strains of epiphytic bacteria achieved differing levels of protection from frost injury although all strains were present at similar population levels.

In addition, infection by *E. amylovora* was greatest at mid-blossom suggesting that plant physiological factors are of importance both in infection and disease control. Host plants are able to influence the populations of associated bacteria, either by producing toxic compounds, which are possibly involved in host plant resistance to phytopathogens (22, 80, 115, 134, 136) or by producing nutritive compounds which sustain epiphytic microbial communities (38, 51, 75, 114). These experiments were carried out with excised apple blossom at various stages throughout blossoming. Major metabolic changes occur in senescing flowers with a rapid increase in respiration and hydrolysis of protein and carbohydrate cell components, dictated by changes in plant hormones (64). Therefore, as blossoming progresses cellular constituents and plant products available to bacteria associated with the plant can change rapidly and dramatically. Such changes in the biotic environment can cause changes in the balance between interacting epiphytic bacteria, possibly influencing the varying pathogenicity and varying disease control observed in these investigations.

CHAPTER 5

ANTIBIOTIC PRODUCTION BY Eh1087

SUMMARY

Eh1087 produces a small (< 10,000 M. Wt.), non-peptide, β-lactam antibiotic *in vitro*. The presence of excess ferric ions or excess essential amino acids does not inactivate the antibiotic, although excess histidine inhibits growth of Eh1087, thereby reducing the antibiotic effect on Ea8862. The antibiotic is pH stable. Cell-free broth supernatants of Eh1087 containing antibiotic activity reduce severity of symptom expression in immature pear fruit.

INTRODUCTION

E. herbicola produces both antifungal (81, 175) and antibacterial (13, 74, 163, 177, 178) activities, with single strains sometimes producing more than one bacteriocin or antibiotic (74, 175). A number of strains producing antibiotics active against *E. amylovora* have been investigated and the role of these antibiotics in biological control has been examined. Mutants of *E. herbicola* defective in antibiotic production have reduced ability to control disease (78, 161) and partially purified antibiotic fractions have been shown to reduce the severity of symptom expression (this study, 74, 177).

The antibiotics produced by the strains of *E. herbicola* so far examined are clearly different compounds with differing spectra of activity and different sensitivities to

various inactivating agents. However, some similarites do exist, suggesting possible relationships. The antibiotics are all small molecules, with reported sizes ranging from 296 d (177) to less than 3,500 d (74).

Ishimaru *et al.* (74) suggest that herbicolin I of *E. herbicola* C9-1 may be related to herbicolicin 112Y, the bacteriocin of *E. herbicola* 112Y, as mutants of *E. amylovora* resistant to herbicolin I are also resistant to herbicolicin 112Y. In addition, both activities are protease resistant, heat stable, labile to base and share similar spectra of activity.

Histidine inactivation of *E. herbicola* antibiotic activity against *E. amylovora* is frequently observed (44, 74, 163, 178), suggesting that *E. herbicola* strains commonly produce an antibiotic, or a group of related antibiotics, that interferes with histidine biosynthesis in *E. amylovora*. Histidine and arginine inactivate the antibiotic of *E. herbicola* Eh318, which has been found to be a competitive inhibitor of N-acetylornithine transaminase, an enzyme involved in arginine biosynthesis (178). This compares with phaseolotoxin, a phytotoxic antibiotic produced by *Pseudomonas phaseolicola*, which shows a similar molecular structure to the antibiotic of Eh318 and which competitively inhibits ornithine carbamyl transferase, another enzyme of the arginine biosynthetic pathway (122).

In this study the antibiotic of Eh1087 is partially characterised. Cell-free culture supernatants of Eh1087, showing antibiotic activity are demonstrated to show some reduction in disease expression in immature pear fruits.

METHODS AND MATERIALS

Broth culture

Cell-free culture supernatants with antibiotic activity were obtained from overnight (12-16 hours) HSN broth cultures supplemented with a 1:50 dilution of pear homogenate, prepared as follows: An equal mass of immature pears (cv. Bartlett) and distilled water were homogenised in a Waring blender and strained through cheesecloth. The strained liquid was centrifuged 15 minutes at 9,000 rpm in a Sorvall GSA rotor to sediment debris. The supernatant was clarified by filtration (0.45 μm, Millipore) and autoclaved prior to use.

Partial purification of antibiotic

Activity in cell-free broth supernatants (pH adjusted to pH 7 with 2N KOH) was adsorbed onto C18 resin (pre-washed in 100% MeOH, 50% MeOH and distilled water) and eluted with a stepwise 10% - 100% MeOH gradient. Activity eluted at 20% MeOH. Fractions were rotary evaporated and redissolved in sterile distilled water to give a 5-fold concentration. Concentrated fractions were assayed for activity by a drop plate method in which 20 μl volumes were dropped onto a freshly prepared soft agar overlay lawn of Ea8862 on HSN agar. Plates were incubated overnight and examined for zones of inhibition of Ea8862 growth.

Gel filtration of ten-fold concentrated broth supernatants was performed on Bio-Gel P2 (100-200 mesh, BioRad). Concentrated supernatant (2 ml) was loaded onto a 20 ml column and eluted in 100 mM ammonium bicarbonate buffer at a flow rate of 1 $ml.min^{-1}$. Fractions eluted off the column were tested for antibiotic activity using the drop plate method.

Batch adsorption to DEAE-sephadex (Pharmacia) in Tris-HCl buffer, pH 5 - 9 and CM-sephadex (Pharmacia) in sodium citrate buffer, pH 4 - 8, was tested according

to the manufacturers instructions. Column chromatography on Dowex 50W and Amberlite resins (Sigma), pre-cycled with 1M NaOH and 1M HCl, was carried out. Columns were eluted in 5% ammonia solution.

Inactivation of the antibiotic

HSN agar plates containing excess trypsin (20 $\mu g.ml^{-1}$ final concentration), $FeCl_3$ (20 $\mu g.ml^{-1}$ final concentration) or essential amino acids (24 $\mu g.ml^{-1}$ final concentration) were prepared with overlay lawns of Ea8862 and spot inoculated with Eh1087, to determine the effect of the added substances on the antibacterial activity of Eh1087. Plates were incubated overnight at 30°C and examined for zones of inhibition the next day.

Inactivation of antibacterial activity in culture supernatant fractions was tested using the drop plate method as above. Aliquots of active cell-free broth supernatants of Eh1087 were incubated 1 hour at 37°C with β-lactamase (Type 1; *Bacillus cereus*) at a final concentration of 0.1 - 2.0 $mg.ml^{-1}$ in 0.125 M citrate buffer pH 7.0. Active supernatant fractions eluted off a C18 column (partially purified antibiotic) were incubated 30 minutes at 37°C with proteinase K (100 $\mu g.ml^{-1}$ final concentration in 10 mM Tris pH 7.8, 5 mM EDTA, 0.5% SDS).

All enzymes and amino acids were supplied by Sigma Chemical Co., (USA).

pH stability

Aliquots of active cell-free broth supernatants of Eh1087 (pH 4.0) were pH adjusted to pH 7.0 and 9.0 with 2N KOH, or left unadjusted, and incubated 1 hour at 37°C. Fractions were then re-adjusted to pH 7.0 and tested for inactivation using the drop plate method.

Molecular weight estimation

An HSN agar plate was overlaid with moist, sterile dialysis membrane (M. Wt. cut-off 10,000 d) and toothpick inoculated with single colonies of Eh1087. Plates were incubated overnight at 30°C. The next day the dialysis membrane carrying Eh1087 growth was removed and the plate overlaid with a soft-agar overlay lawn of Ea8862 and re-incubated. The following day the plate was examined for zones of inhibition of Ea8862. Alternatively, 20 μl volumes of sterile culture supernatant were dropped onto an HSN plate overlaid with dialysis membrane. Once the liquid had absorbed into the agar the dialysis membrane was removed and a soft agar overlay of Ea8862 was poured.

Induction of antibiotic

Mitomycin C (Sigma) was incorporated into HSN agar plates at a final concentration of 0.5 $mg.ml^{-1}$ or 1.0 $mg.ml^{-1}$ according to the method of Pugsley and Oudega (127). Overlay lawns of Ea8862 were inoculated with Eh1087 and incubated as usual. Overlay lawns of Eh1087 and Ea8862 alone with mitomycin C were used as controls. All plates were examined for zones of inhibition.

Immature pear fruit assay

Cell-free culture supernatants were co-inoculated with Ea8862 onto immature pear fruit as previously described (Chapter 3).

RESULTS

Antibiotic production by Eh1087 in HSN broth culture was erratic, but was stimulated by addition of a sterile pear homogenate to the medium. Addition of the pear homogenate seemed to improve the buffering capacity of the liquid medium as the pH of broth cultures containing pear homogenate never dropped below pH 6.0, whereas broth cultures lacking pear homogenate frequently dropped

to pH 4.0. This acidification was never seen on solid HSN medium; the pH of inhibition zones on agar plates was never lower than pH 5.5.

The antibiotic activity of Eh1087 was not inactivated by $FeCl_3$, indicating that a siderophore activity is not involved. Trypsin added to the HSN medium in excess, caused a 50% reduction in the size of inhibition zones of Ea8862. However, proteinase K digestion of partially purified antibiotic caused no reduction of activity. Therefore it seems likely that the trypsin may have been acting on Ea8862, rather than on Eh1087, possibly by modifying antibiotic receptor protein(s) on the surface of Ea8862 cells.

The inhibition zones produced by Eh1087 in the presence of mitomycin C were no larger than those produced in the absence of mitomycin C, indicating that the antibiotic is not inducible.

Antibiotic production was evident on complex nutrient medium (LB agar) and none of the essential amino acids individually inactivated the antibiotic, although excess histidine in the HSN medium inhibited growth of the producer strain, thereby reducing inhibition of Ea8862 (Figure 5.1).

β-lactamase digestion, at a concentration of 1 $mg.ml^{-1}$, destroyed antibiotic activity in cell-free culture supernatants, indicating that Eh1087 produces a β-lactam antibiotic (Figure 5.2).

The antibiotic of Eh1087 passed through dialysis membrane, indicating a molecular weight of less than 10,000 d. When concentrated culture supernatant was filtered through a Bio-Gel P2 column antibacterial activity eluted in the void volume, indicating a molecular weight greater than 1,800 d and beyond the fractionation limit of the resin used.

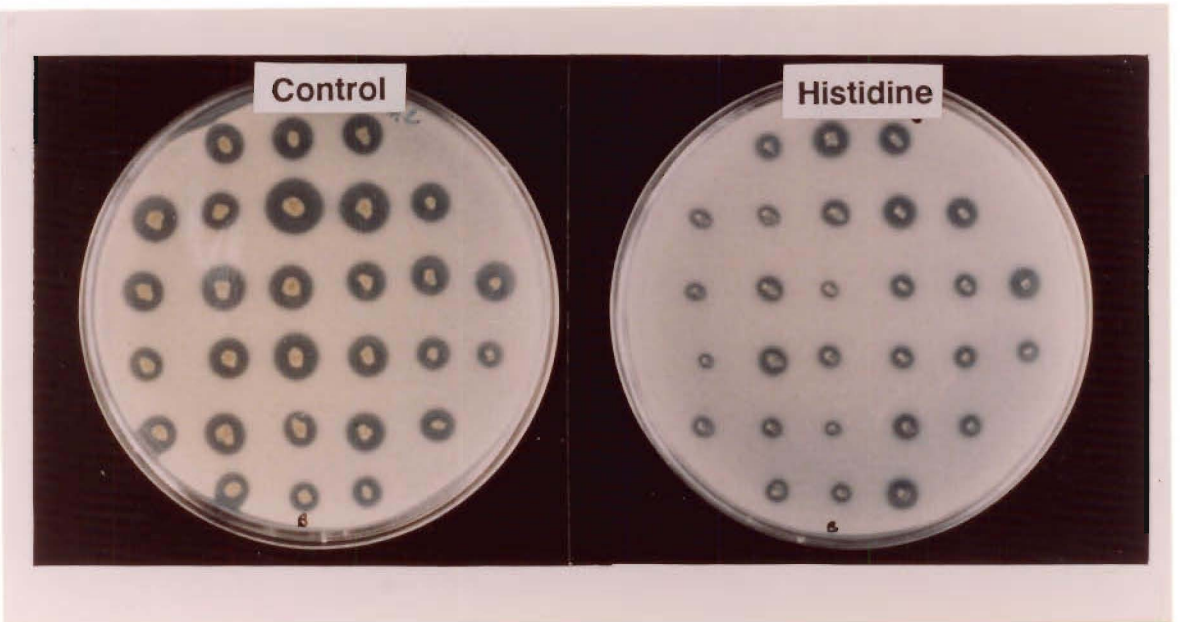


Figure 5.1: Inhibition of the antibiotic activity of Eh1087 by histidine. Histidine added to HSN agar (at right) reduces growth of Eh1087 leading to reduced inhibition of Ea8862.

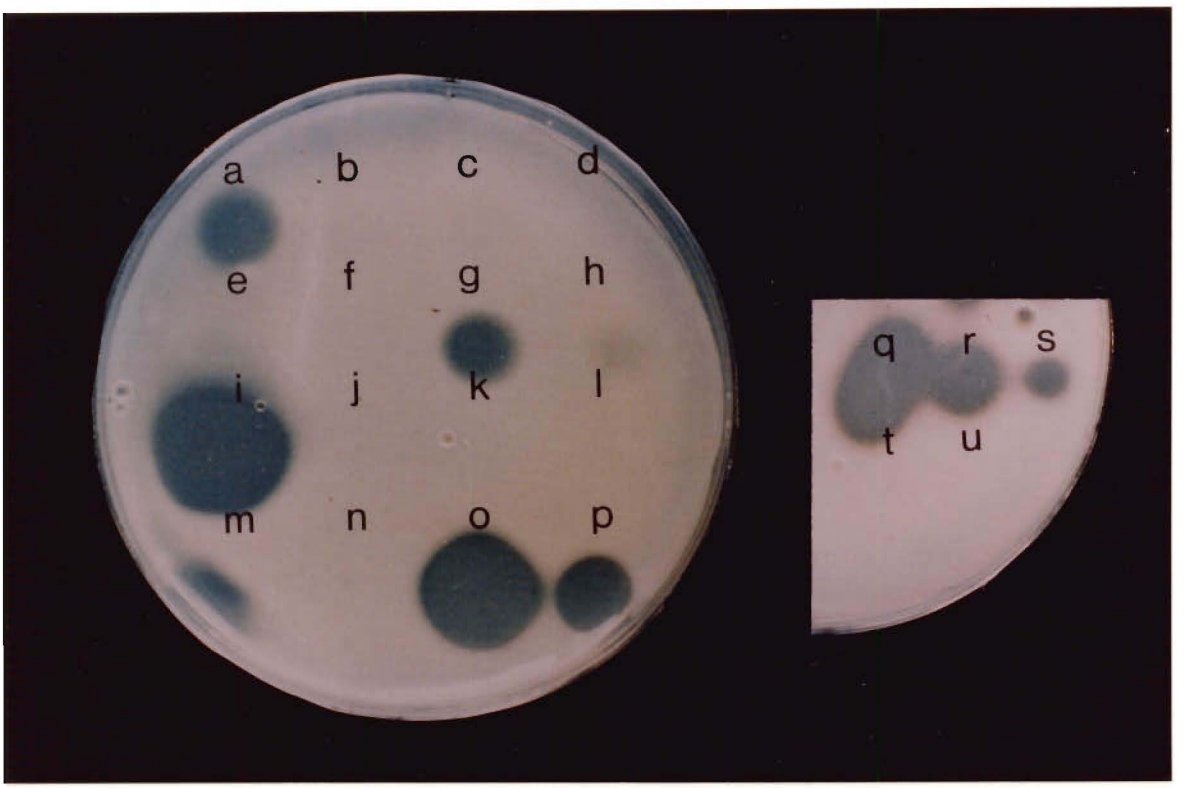


Figure 5.2: Inactivation of the antibiotic activity of Eh1087 by β-lactamase. a) Eh1087 HSN culture supernatant, b) HSN broth, c) citrate buffer, d) β-lactamase, e) penicillin G, f) penicillin G + β-lactamase, g) Eh1087 culture supernatant, h) Eh1087 culture supernatant + β-lactamase, j) - p) as for a) - h) but with a 5-fold concentration of Eh1087 culture supernatant and penicillin, q) Eh1087 culture supernatant (5-fold conc.), r) - u) Eh1087 culture supernatant (5-fold conc.) + β-lactamase at 100 $\mu g.ml^{-1}$, 200 $\mu g.ml^{-1}$, 1,000 $\mu g.ml^{-1}$ and 2,000 $\mu g.ml^{-1}$, respectively.

Immature pear fruit co-inoculated with Ea8862 and cell-free culture supernatants of Eh1087 showed a reduction in the severity of symptom expression compared to that in pears inoculated with Ea8862 alone, although actual numbers of infected pear slices were not reduced.

Antibacterial activity did not bind to weak anion (DEAE-sephadex) or cation exchangers (CM-sephadex) and bound irreversibly to a strong cation exchanger (Dowex 50W) and a mixed bed resin (Amberlite). Reversed phase chromatography with C18 resin allowed a partial purification. Active fractions were eluted in 20 % MeOH, rotary evaporated and re-dissolved in sterile distilled water.

DISCUSSION

Unreliable production of antibiotic activity in broth cultures hampered purification and characterisation studies. Bacteriocins and antibiotics are generally produced on solid rather than liquid media and very small changes in culture conditions can cause substantial changes in antibiotic/bacteriocin yield when using broth culture (103). Addition of plant tissue (pear homogenate) stimulated antibiotic production, suggesting that the antibacterial effect of Eh1087 may be host induced. Host induction of antibacterial activity in Eh1087 may parallel the host induction of a plant toxin in *E. amylovora* (34, 185).

Mitomycin C induction of antibacterial activity was investigated as a possible means to obtain higher antibiotic yields and to overcome the problems caused by variable yields from non-induced broth cultures. Furthermore, induced cultures can be harvested after a shorter incubation time thereby avoiding any cell autolysis

products that occur in aged cultures and that can interfere with antibiotic expression. However, incorporation of mitomycin C into solid media did not induce antibacterial activity in Eh1087.

The antibiotic of Eh1087 did not bind to weak ion exchangers and bound irreversibly to strong ion exchange resins. This is in contrast to other *E. herbicola* antibiotics which have been purified using strong ion exchangers, in conjunction with gel permeation chromatography (74, 143). Reversed phase chromatography enabled a partial purification and further RP-HPLC is planned to continue purification of the antibiotic.

The antibiotic of Eh1087 is a non-peptide, non-siderophore activity that is not inhibited by any of the essential amino acids. Non-peptide antibiotics are also produced by *E. herbicola* strains EhC9-1 (74) and Eh112Y (13), although these antibiotics are clearly not related to that of Eh1087 as they differ in spectra of activity, size, pH stability and sensitivity to inactivation by amino acids (13, 74, 143, 178). Siderophore activity has been implicated in the virulence of *E. amylovora* (141, 159), but has not been reported to contribute to the inhibitory activity of *E. herbicola*.

Antibiotics produced by *E. herbicola* are often inactivated by the addition of certain essential amino acids to the medium (44, 177). Half (3/6) the characterised antibiotics of *E. herbicola* are inhibited by histidine in the medium (74, 163, 178). In a recent survey of *in vitro* antibiotic producing strains of *E. herbicola* (44), 37% were inactivated by added histidine, suggesting that antibiotics inactivated by histidine are typically produced by *E. herbicola*. Excess histidine in the medium reduced overall growth of Eh1087, thereby reducing the size of inhibition zones produced against Ea8862. However, it did not inhibit antibacterial activity itself. This was confirmed by drop plate assays of sterile culture supernatants and histidine, showing no loss of inhibition of Ea8862.

β-lactamase digestion inactivated the Eh1087 antibiotic. Partial inactivation of the antibiotic was seen when β-lactamase concentrations below 1 $mg.ml^{-1}$ were used. Type 1 β-lactamase has both penicillinase activity and a low level of cephalosporinase activity (1,500-3,000 $U.mg^{-1}$ penicillinase and 10-30 $U.mg^{-1}$ cephalosporinase). As bacteria do not produce penicillins (3), this indicates that the antibiotic of Eh1087 is susceptible to cephalosporinase. The low level of cephalosporinase activity in type 1 β-lactamase probably explains the need to use high levels of the enzyme to completely inactivate the Eh1087 antibiotic.

Bacteria producing monocyclic β-lactam antibiotics were discovered about ten years ago and are relatively common in nature (148). In addition, *E. herbicola* has been reported to produce a novel bicyclic β-lactam antibiotic with a broad spectrum of activity *in vitro* (123). Research into the chemistry and biology of bacterial β-lactam antibiotics is largely conducted to search for clinically useful compounds and the activity of these antibiotics against bacteria non-pathogenic to humans is not known. To the authors knowledge, this is the first report of a β-lactam antibiotic produced by *E. herbicola*, with activity against *E. amylovora*.

Cell-free supernatants of Eh1087 broth cultures with *in vitro* antibacterial activity reduced the severity of disease symptom expression in the IPFA, although no reduction in the number of diseased pears was seen. The antibiotics of Eh318 and EhC9-1 also showed ability to reduce disease severity in immature pears (177 and 74, respectively). Demonstration that *E. herbicola* antibiotics are active *in planta* clearly indicates a role for antibiosis in disease control, as has been suggested by other researchers (13, 163 and 177).

CHAPTER 6

TRANSPOSON MUTAGENESIS OF Eh1087

SUMMARY

Tn*phoA*-insertion mutants of Eh1087 were created (Ant$^-$), which failed to produce antibiotic activity *in vitro* and which failed to inhibit Ea8862 in immature pear fruit. Fifty percent (6/12) of the mutants obtained were shown to have single Tn*phoA* insertions by Southern hybridisation. Restriction mapping of mutant fragment clones and southern hybridisations indicated that the insertions were located within a 1.5 kb region of DNA on a 200 kb indigenous plasmid of Eh1087.

INTRODUCTION

To investigate the molecular basis of inhibition of *E. amylovora* by *E. herbicola*, mutants with altered inhibitory capability are required. To date, mutagenesis of inhibitory *E. herbicola* strains has enabled elucidation of the role for antibiosis in disease control (13, 55, 163). The use of mutants of *E. amylovora*, with altered sensitivity to the antibiotics of *E. herbicola*, has also proved useful (74, 161). Genes coding for antibiotic expression have been located on plasmids (this study, 55) and in the chromosome (163) of *E. herbicola* and genetic analysis of these loci is currently under way (this study, 163).

In this study Tn*phoA* mutagenesis was used to generate mutants of Eh1087 defective in antibiotic production. Use of Tn*phoA* can facilitate selection for mutations in genes for secreted products, such as antibiotics, through the formation of hybrid proteins with alkaline phosphatase activity (100).

Tn*phoA* is a derivative of Tn5 which carries the structural gene for alkaline phosphatase, lacking its signal sequence, in the IS50L sequence (100). Tn*phoA* insertion results in a gene fusion between the *phoA* gene and the gene into which the transposon has inserted. If Tn*phoA* inserts into a gene coding for a secreted peptide or protein, active hybrid proteins can result, in which alkaline phosphatase is secreted as part of a hybrid protein, using the signal sequence at the amino terminal of the target peptide/protein. The alkaline phosphatase enzyme is only active extracellularly, so only those hybrid proteins that are secreted from the cytoplasm have alkaline phosphatase activity. Selection for these mutations is easily carried out using a chromogenic substrate for the enzyme incorporated into the selective medium.

In this study, the phenotypic characteristics of the Ant$^-$ Tn*phoA*-insertion mutants were investigated and the sites of Tn*phoA* insertion mapped.

Bacterial Strains and Plasmids Used

Bacterial strains and plasmids used are shown in Table 6.1.

Table 6.1: Bacterial strains and plasmids

Strain or Plasmid	Characteristics[a]	Reference or source
Erwinia amylovora		
Ea8862	isolated from *Malus* X. *domestica*	ICMP[b]
Erwinia herbicola		
Eh1087	Ap^R Rif^R	This study
EhA12e	Km^R Ant^- mutant	" "
EhA14b	" " "	" "
EhA17g	" " "	" "
EhA19f	" " "	" "
EhA20g	" " "	" "
EhA46a	" " "	" "
Escherichia coli		
DH5α	*endA hsdR supE thi gyrA* U169ϕ80d *relA lac* ZΔM15 Δ(*argf-lacZYA*)	BRL[c] (19)
KS300	MC10000 *RecA* Δ(*phoA-pvuII*)	Supplied by J. Beckwith
SM10	*thi thr leu tonA lacY supE RecA*::RP4-2-Tc::Mu:: (λpir^+)	Simon *et al.* (138)
MM294	*endA hsdR pro supF*	Ruvkun and Ausubel (131)
Plasmid		
pRT291::Tn*phoA*	Tc^R Km^R IncP-1	Taylor *et al.* (149)
pPH1JI	Gm^R Str^R Spc^R IncP-1	Beringer *et al.*(18)
pRT733::Tn*phoA*	oriR6K Ap^R Km^R	Taylor *et al.* (149)
pUC19	Ap^R *LacZ*α$^+$	Yannisch-Perron *et al.* (184)

[a] Ap^R, Km^R, Gm^R, Str^R and Spc^R indicate resistance to ampicillin, kanamycin, gentamycin, streptomycin and spectinomycin, respectively.
[b] International Collection of Micro-organisms from Plants, Landcare/ Manaaki Whenua Research New Zealand Ltd., Auckland, New Zealand
[c] Bethesda Research Laboratories

Enzymes, antibiotics and substrates

All restriction enzymes and buffer solutions were obtained from Bethesda Research Laboratories (BRL). Restriction enzymes were used at a concentration of 3-5 U.μg^{-1} DNA. Antibiotics and 5-bromo-4-chloro-3-indolyl phosphate (XP) were obtained from Sigma Chemical Co. (USA) and were used at the following concentrations: rifampicin (Rif) 50 $\mu g.ml^{-1}$, kanamycin (Km) 50 $\mu g.ml^{-1}$, gentamycin (Gm) 30 $\mu g.ml^{-1}$, tetracycline (Tc) 15 $\mu g.ml^{-1}$ and XP 40 $\mu g.ml^{-1}$. Bacterial alkaline phosphatase (BAP) was obtained from Gibco BRL and was used at a concentration of 1U.μg^{-1} DNA. T4 DNA ligase and ligase buffer were obtained from Boehringer Mannheim.

Conjugational mutagenesis of Eh1087

For mutagenesis, a spontaneously arising Rif^R variant of Eh1087 was used. Two alternative plasmid vector systems for Tn*phoA* delivery into Eh1087 were used. Both methods were according to Taylor *et al.* (149).

The first method involved two consecutive conjugations. In the first conjugation, Tn*phoA* vector plasmid pRT291 (Tc^R) was mobilised into Eh1087. In the second conjugation, pPH1JI (Gm^R), a plasmid belonging to the same incompatibility group as pRT291, was introduced into exconjugants from the first mating. Conjugation conditions were as described below. Selection for Km^R, Gm^R, Tc^S colonies selected for exconjugants in which the pRT291 plasmid vector had been lost, due to plasmid incompatiblity, with concommittant transposition of Tn*phoA*.

In the second method, Tn*phoA* was mobilised into Eh1087 using a suicidal vector plasmid, pRT733 (Ap^R), which cannot replicate without the *λpir* gene product (84), which is provided in *trans* in the *E. coli* donor strain, but which is absent in Eh1087.

Equal volumes (0.5 ml) of overnight LB broth cultures of the donor strain, *E. coli* SM10(pRT733::Tn*phoA*), and Eh1087 (Rif^R) were mixed and the cells sedimented by centrifugation at 12,000 rpm for 2 minutes. Cells were gently resuspended in

200 μl LB broth and deposited onto a sterile 0.22 μm filter (Millipore) on a pre-warmed LB agar plate and incubated 7-8 hours at 25°C. After incubation cells were resuspended in 5 ml sterile 0.85% saline (w/v) and 100 μl plated out onto LB supplemented with Rif, Km and XP. Plates were incubated overnight at 30°C. Control plates of *E. coli* and Eh1087 on the selective medium were also included.

Confirmation of Suicide of Vector Plasmid, pRT733

Loss of the vector plasmid pRT733 in Rif^R, Km^R exconjugants could not be confirmed by screening for loss of its Ap^R marker as the wild type Eh1087 was also resistant to ampicillin. Therefore, plasmid mini-preparations were made for each Ant^- mutant according to the method of Sambrook *et al.* (133) and electrophoresed in 0.8% agarose. Absence of plasmid DNA bands in each mutant strain confirmed loss of the pRT733 plasmid.

Screening for Loss of Inhibition of Ea8862

Individual colonies of exconjugants growing on the selective medium were toothpicked onto HSN agar plates seeded with a soft agar overlay lawn of Ea8862 (see Chapter 3 for method) and incubated overnight at 30°C. Colonies failing to give inhibition zones were re-streaked and re-assayed to confirm colony purity and mutation stability. Mutants failing to inhibit Ea8862 on HSN agar were also tested for inhibition of Ea8862 in the immature pear fruit assay (see Chapter 3 for method) and for production of antibiotic activity in cell-free culture supernatants (see Chapter 5 for method).

Screening for Auxotrophic Mutants

Tn*phoA*-insertion mutants were screened for auxotrophy on M63 minimal medium, with added vitamin B1 and using glucose as the carbon source (108). Individual colonies were resuspended in 10 mM $MgSO_4$ and then transferred to M63 medium to avoid possible carry-over of nutrients from the LB selection plates.

Rate of growth of Ant⁻ mutants

The rate of growth of Ant⁻ mutant strains in HSN broth and in immature pear fruit was compared with that of wild-type Eh1087.

HSN broth (10 ml) was inoculated with 200 μl of an overnight LB broth, supplemented with the appropriate antibiotics, of the test strain and the $O.D._{600}$ of a 2 ml aliquot was measured. Broths were incubated 5 hours at 30°C with orbital shaking (200 rpm) and the $O.D._{600}$ was measured again.

Immature pear fruit slices (3 slices per test) were inoculated with 50 μl of an overnight LB broth culture, supplemented with the appropriate antibiotics, of the test strain ($O.D._{600}$ 1:10 dilution = 0.28; approximately 2.5×10^9 $cfu.ml^{-1}$) and incubated 3 days at 20°C in sterile humidity chambers. Pear fruit slices were then washed in 10 ml 0.85% sterile saline (w/v) and the wash was dilution plated on LB agar, supplemented with the appropriate antibiotics.

Screening for loss of immunity to Eh1087 antibiotic

Ant⁻ mutants of Eh1087 were screened for loss of immunity to the Eh1087 antibiotic activity on HSN agar using the streak plate method, as described in Chapter 3. A single central streak of Eh1087, cross-streaked with Ant⁻ mutant strains and with Eh1087 and Ea8862 controls, was made. Zones of inhibition were observed after overnight incubation at 30°C.

DNA Hybridisation: Tn*phoA* Insertion Mutants

Southern blotted DNA from Ant⁻ mutants was hybridised with a Tn*phoA* fragment probe (Figure 6.1) to the number of Tn*phoA* insertions in each mutant. Total DNA was purified using the method of Scott *et al.* (135). DNA (500 ng) was completely digested with *Eco*RV and electrophoresed on 1% agarose. Eh1087 wild type and pRT733::Tn*phoA* plasmid DNA were included as controls. Gels

were stained in ethidium bromide (0.5 $\mu g.ml^{-1}$) and photographed prior to blotting. Alkali blotting onto Hybond N+ (Amersham) membranes was carried out according to the manufacturers instructions, using the Vacublot system (BioRad).

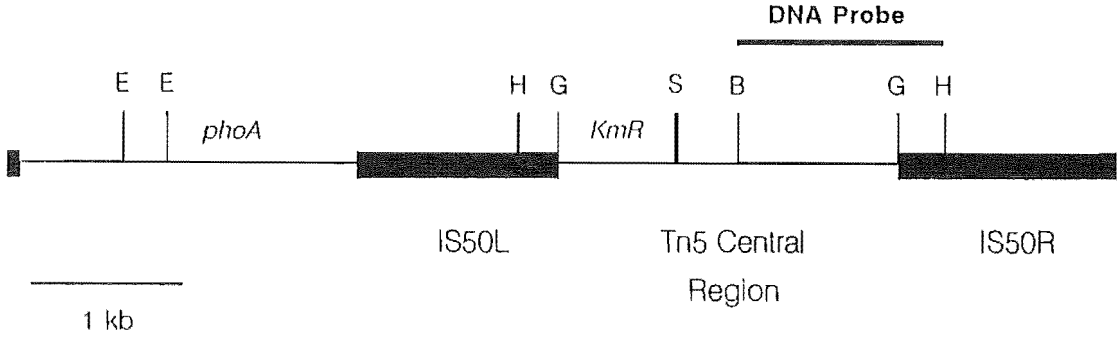


Figure 6.1: Restriction map of Tn*phoA* (courtesy C. Manoil) showing *Bam*H1-*Hin*dIII 1.4 kb fragment probe. E, H, G, S and B indicates restriction sites for *Eco*R1, *Hin*dIII, *Bgl*II, *Sal*1 and *Bam*H1, respectively.

The Tn*phoA* fragment probe was prepared as follows: Tn*phoA* DNA was *Bam*H1-*Hin*dIII digested and electrophoresed in 0.7% agarose. The desired fragment (1.4 kb) was harvested from a gel slice by the method of Heery *et al* (67) and ^{32}P-labelled using the BRL random primers system according to the manufacturers instructions.

Southern blotted DNA was hybridised with the following procedure: Hybond N+ membranes were pre-hybridised for 2-3 hours at 65°C in 0.5M Na_2HPO_4 pH7.2, 1mM EDTA, 7% SDS. ^{32}P-labelled Tn*phoA* fragment probe was added at 200 cpm.membrane^{-1} and hybridised overnight at 65°C. Membranes were washed 30-60 minutes at 65°C in 40 mM Na_2HPO_4 pH7.2, 1 mM EDTA, 1% SDS. Membranes were dried and autoradiographed 6-12 hours at -80°C with Hyperfilm-MP (Amersham) in Cronex intensifying cassettes (DuPont).

Subcloning Mutant Fragments into pUC19

*Bam*H1 digestion of DNA from Ant$^-$ mutants yielded fragments containing the Km^R gene of Tn*phoA* and the IS50L, plus left-hand flanking Eh1087 DNA. These restriction fragments were termed mutant fragments. Mutant fragments from single Tn*phoA*-insertion mutants were subcloned into pUC19 using a shotgun cloning technique as follows: Total DNA from each mutant strain was digested with *Bam*H1 and phenol:chloroform extracted twice. Plasmid pUC19 was linearised by cleavage at the *Bam*H1 site, phenol:chloroform extracted, ethanol precipitated and resuspended in TE (10 mM Tris-HCl pH8, 1 mM EDTA). Resuspended DNA was dephosphorylated with BAP for 60 minutes at 65°C. The linear dephosphorylated DNA was phenol:chloroform extracted and ethanol precipitated as before and the DNA was resuspended to a final concentration of 30 μg.ml^{-1}.

Ligation of *Bam*H1-digested mutant DNA to linear pUC19 was carried out overnight at 12-14°C with DNA mass ratios of 2:1, 4:1 and 10:1 (Ant$^-$ mutant:pUC19). Each ligation used 2.5 U of T4 DNA ligase in a total volume of 20 μl. Controls were included to check the efficiency of linearisation (pUC19 without ligase) and of dephosphorylation of pUC19 (pUC19 with ligase). Appropriate volumes (5 or 10 μl) of each ligation reaction were used to transform competent *E. coli* DH5α.

In addition, *E. coli* KS300 (phoA$^-$) was transformed with two mutant fragment clones obtained from the double insert mutant EhA21a, which produced blue colonies on medium containing XP. KS300 transformed with the clone containing an active *phoA* gene fusion produced blue colonies in the presence of XP.

Preparation of Competent Cells

LB broth (100 ml) was inoculated with 2 ml of overnight culture of *E. coli* DH5α or KS300 and grown at 37°C to O.D.$_{600}$ = 0.6. The broth culture was aseptically transferred to chilled sterile 50 ml plastic centrifuge tubes and centrifuged at 2,000

rpm for 10 minutes in a benchtop centrifuge at 4°C. The supernatant was decanted from the cells and tubes inverted for 1 minute to allow all traces of the LB medium to drain away. Each cell pellet was resuspended in 10 ml ice-cold 50 mM $CaCl_2$ and stored on ice for 20 minutes. Cells were sedimented as before and each pellet resuspended in 2 ml ice-cold 50 mM $CaCl_2$. Cells were stored on ice for 30 minutes to 16 hours before transformation.

Transformation of Competent Cells

Ligated DNA (1,5 or 10 μl of ligation reaction) was added to competent cells (200 μl) in sterile tubes on ice. The contents were mixed by gently swirling and tubes were stored on ice for 30 minutes to 2 hours. Cells were then heat-shocked for exactly one minute at 42°C and cooled immediately on ice. LB broth (1 ml) was added and cells were elaborated for 1 hour at 37°C with gentle or no shaking to allow expression of the acquired antibiotic resistance marker. Cells were sedimented at 4°C and resuspended cell pellets were plated out on LB supplemented with Km for transformed DH5α and with Km and XP for transformed KS300. A control of non-transformed competent cells plated on the selective medium was also included.

Restriction Mapping of Cloned Mutant Fragments

Mutant fragment clones were restriction mapped with *Eco*R1, *Sal*1, *Hin*dIII, *Bam*H1, *Pst*1 and *Sac*1. Plasmid DNA was prepared using the method of Sambrook *et al.* (133) or of Del Sal *et al.* (39).

DNA Hybridisation: Eh1087 Plasmid DNA

Southern blotted Eh1087 plasmid DNA was hybridised with a mutant fragment probe (*Eco*R1-*Sal*1 2.4 kb) derived from mutant fragment clone pLK2 (Figure 6.5) to identify whether the mutated site was chromosomal or plasmid-borne. The mutant fragment probe was purified and radio-labelled in the same way as described above for the Tn*phoA* fragment probe. Plasmid preparations were made

according to the method of Comai and Kosuge (32). Electrophoresis was carried out in 0.3% agarose for 36 hours at 1 $V.cm^{-1}$ at 4°C. A strain of *Agrobacterium tumefaciens*, carrying two plasmids of 200 kb and >300 kb, was included as a control (Supplied by B. Palmer, University of Canterbury). Alkali blotting and hybridisation were carried out as described above.

RESULTS

Tn*phoA* mutagenesis of Eh1087 was attempted using two vector delivery systems. Initially, a two plasmid transfer system, using plasmid vector pRT291 and an incompatible plasmid, pPH1JI, (149) was used. However, in Eh1087 both plasmids survived in 100 % of exconjugants for at least two subcultures. For this reason, an alternative Tn*phoA* delivery system, using a suicidal plasmid vector, pRT733, (149) was used to generate mutants of Eh1087.

Twelve Eh1087 Tn*phoA*-insertion mutants (Ant^-), which failed to inhibit Ea8862 *in vitro* (Figure 6.2) were obtained from 8 independent filter matings, with a frequency of transposition of 1×10^{-6}. No spontaneously Km^R mutants of Eh1087 were seen (frequency less than 1×10^{-9}) and no auxotrophic mutants were obtained (800 Km^R colonies screened).

More than one mutant colony was selected from an individual mating when mutant phenotypes differed with respect to pigmentation and/or extracellular polysaccharide production. Ant^- mutant colonies had a white or orange pigmentation on LB agar, in contrast to the yellow pigmentation of wild-type Eh1087. In addition, the mutant colonies were frequently more fluidal than those of the wild-type.

DNA hybridisation of Southern blotted mutant DNA with a Tn*phoA* fragment probe confirmed single Tn*phoA* insertions for 6/12 Ant^- mutants and double insertions for the remaining mutants (Figure 6.3). Two of the double

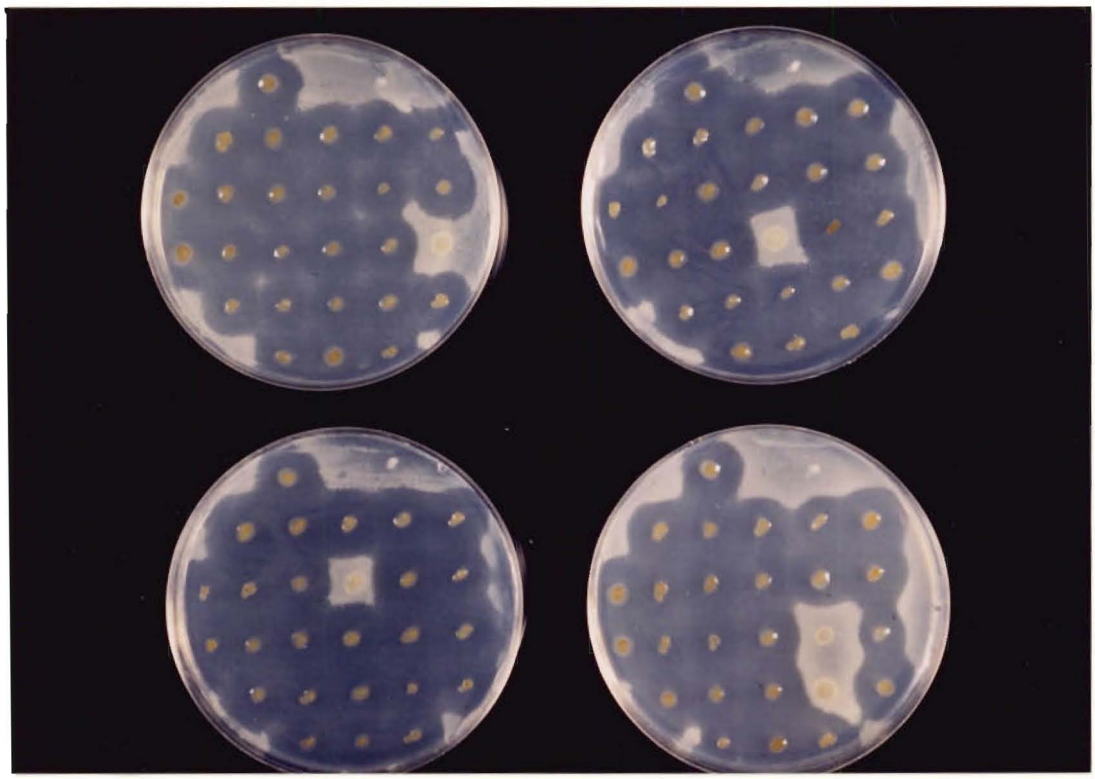


Figure 6.2: Screening for Ant$^-$ mutants of Eh1087 on HSN agar. Transconjugants which fail to inhibit Ea8862 (Ant$^-$) are detected by overgrowth of the Ea8862 bacterial lawn. Controls on the top row of each plate, from left to right, are: Eh1087, *E. coli* SM10(pRT733::Tn*phoA*) and Ea8862.

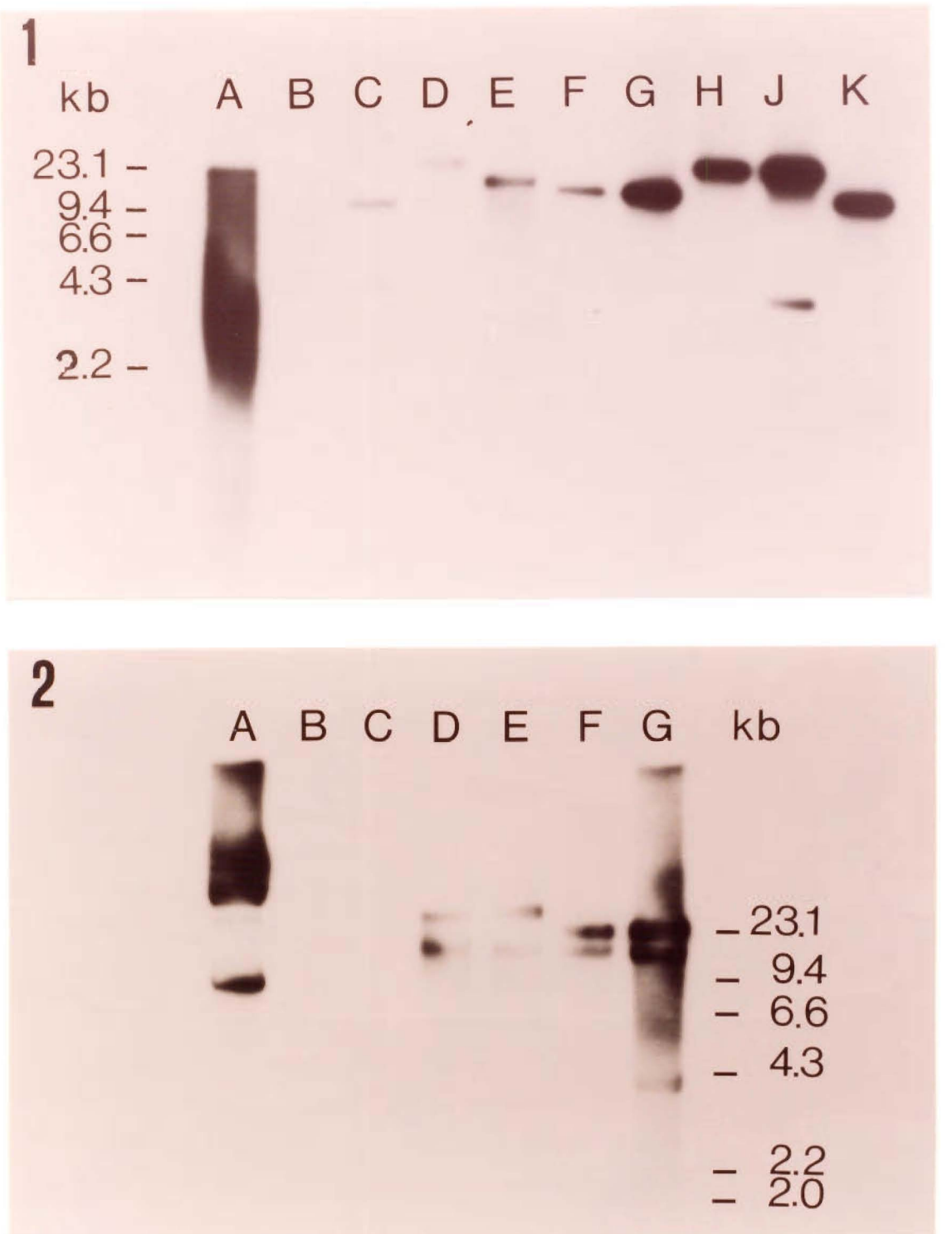


Figure 6.3: Southern hybridisation analysis of *Eco*RV-digested DNA from Ant⁻ mutants (EhA strains).
1: A) pRT733::Tn*phoA*, B) Eh1087, C) EhA45a, D) EhA46a, E) EhA11g, F) EhA17g, G) EhA20g, H) EhA19f, J) EhA10e, K) EhA12e.
2: A) pRT733::Tn*phoA* (undigested DNA), B) Eh1087, C) empty, D) EhA21a, E) EhA24f F) EhA14b, G) EhA18d.
Molecular weight markers are indicated on the left.

insertion mutants, EhA10e and EhA45a, showed DNA bands less than 4 kb hybridising with the Tn*phoA* fragment probe. *Eco*RV digestion of DNA from Tn*phoA*-insertion mutants yields restriction fragments containing the complete inserted Tn*phoA* molecule as this transposon contains no *Eco*RV sites. The size of the Tn*phoA* molecule is 7.6 kb, so the presence of hybridising bands smaller than 7.6 kb suggests that the IS50 sequences have independently transposed in these mutants.

Because of the difficulty in isolating the mutation of interest in mutants carrying double Tn*phoA* insertions, further investigations were concentrated on 5 of the single insertion mutants. These mutants failed to suppress fire blight disease in immature pear fruit (Table 6.2; Figure 6.4) and did not produce any antibiotic activity against Ea8862 in cell-free HSN culture supernatants. Rates of growth of Ant$^-$ mutants did not differ from that of wild-type Eh1087 in HSN broth (Table 6.3) and in immature pear fruit (Table 6.4). Ant$^-$ mutants remained immune to the antibiotic of Eh1087 *in vitro*.

Table 6.2: Immature pear fruit assay of Ant$^-$ mutants

Treatment	% Infection 3 days post inoculation		
	Assay 1	Assay 2	Assay 3
Saline control	0	0	0
Ea8862 control	90	100	100
Ea8862 + Eh1087	20	30	0
Ea8862 + EhA12e	100	80	60
Ea8862 + EhA17g	100	100	60
Ea8862 + EhA19f	100	100	100
Ea8862 + EhA20g	100	90	100
Ea8862 + EhA46a	100	100	100

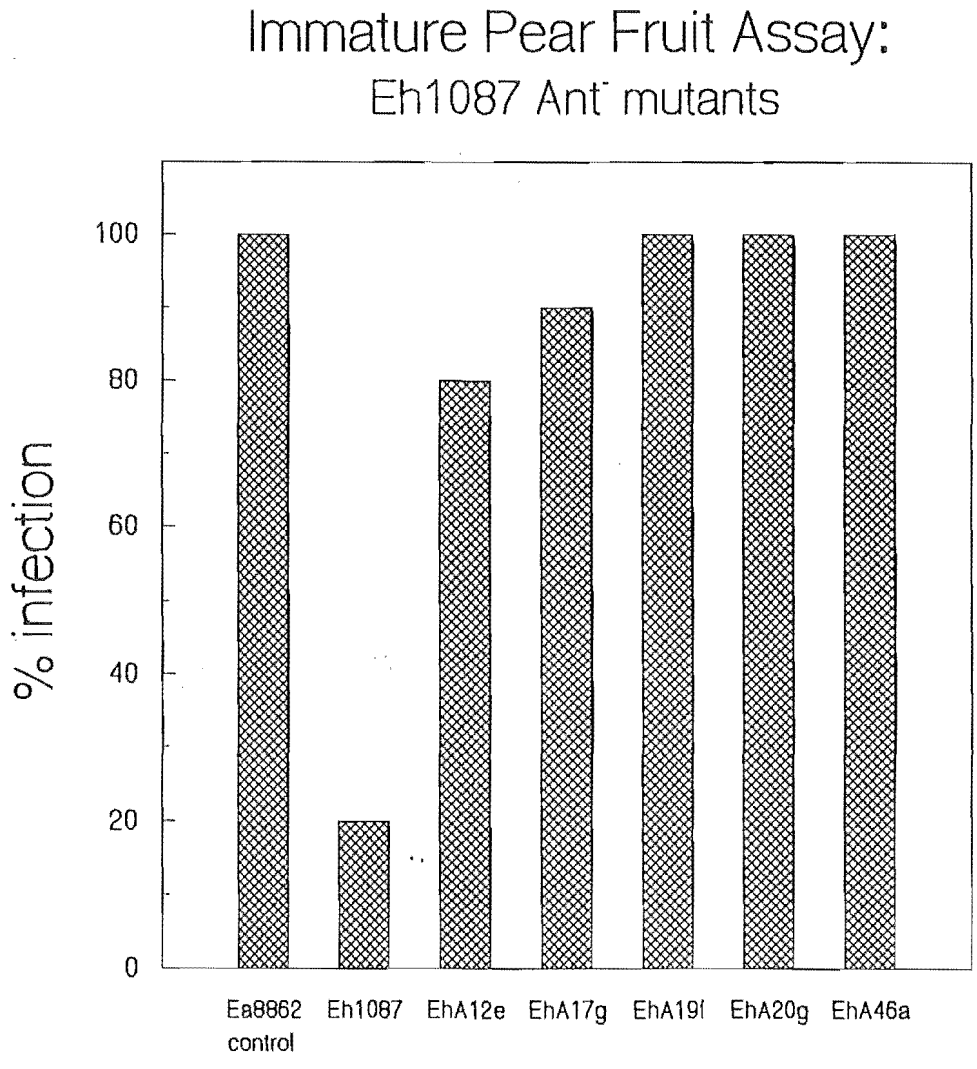


Figure 6.4: Immature pear fruit assay of Ant⁻ mutants of Eh1087. From left to right, % infection in immature pear fruit slices 3 days following inoculation with Ea8862 alone, Ea8862 and Eh1087, Ea8862 and Ant⁻ mutants EhA12e, EhA17g, EhA19f, EhA20g and EhA46a, respectively, is shown. Accumulated data from 3 separate bioassays (Table 6.2) are shown.

Table 6.3: Rates of growth of Ant⁻ mutants and wild-type Eh1087 in HSN broth.

Strain	A_{600nm} Start	$A_{600\ nm}$ Finish	R.O.G. (O.D. units.hr^{-1})
Eh1087	0.12	0.38	0.05
EhA12e	0.13	0.37	0.05
EhA17g	0.12	0.38	0.05
EhA19f	0.11	0.36	0.05
EhA20g	0.11	0.34	0.05
EhA46a	0.11	0.37	0.05

Table 6.4: Rates of growth of Ant⁻ mutants and Eh1087 in immature pear fruit.

Strain	Viable bacteria on pear slices 3 days post inoculation (cfu.ml^{-1})
Eh1087	1×10^8
EhA12e	2×10^8
EhA17g	1×10^8
EhA19f	8×10^7
EhA20g	5×10^7
EhA46a	1×10^8

Mutant fragments, containing recipient Eh1087 DNA flanking the Tn*phoA* insertion site, were generated for each mutant by *Bam*H1 digestion and cloned into pUC19. Restriction maps of these clones are shown in Figure 6.5. In addition, the two mutant fragments from one of the double insertion mutants, EhA21a, which produced blue colonies on medium containing XP, were cloned. The mutant fragment clone that contained the active *phoA* gene fusion was identified by transformation of *E. coli* KS300 (phoA⁻) and selection for blue colonies. This clone, pLK14, was also restriction mapped (Figure 6.6).

Restriction maps of the mutant fragment clones showed overlapping regions, enabling the Tn*phoA* insertion sites for the corresponding mutants to be mapped within a 1.5 kb region of DNA (Figure 6.7).

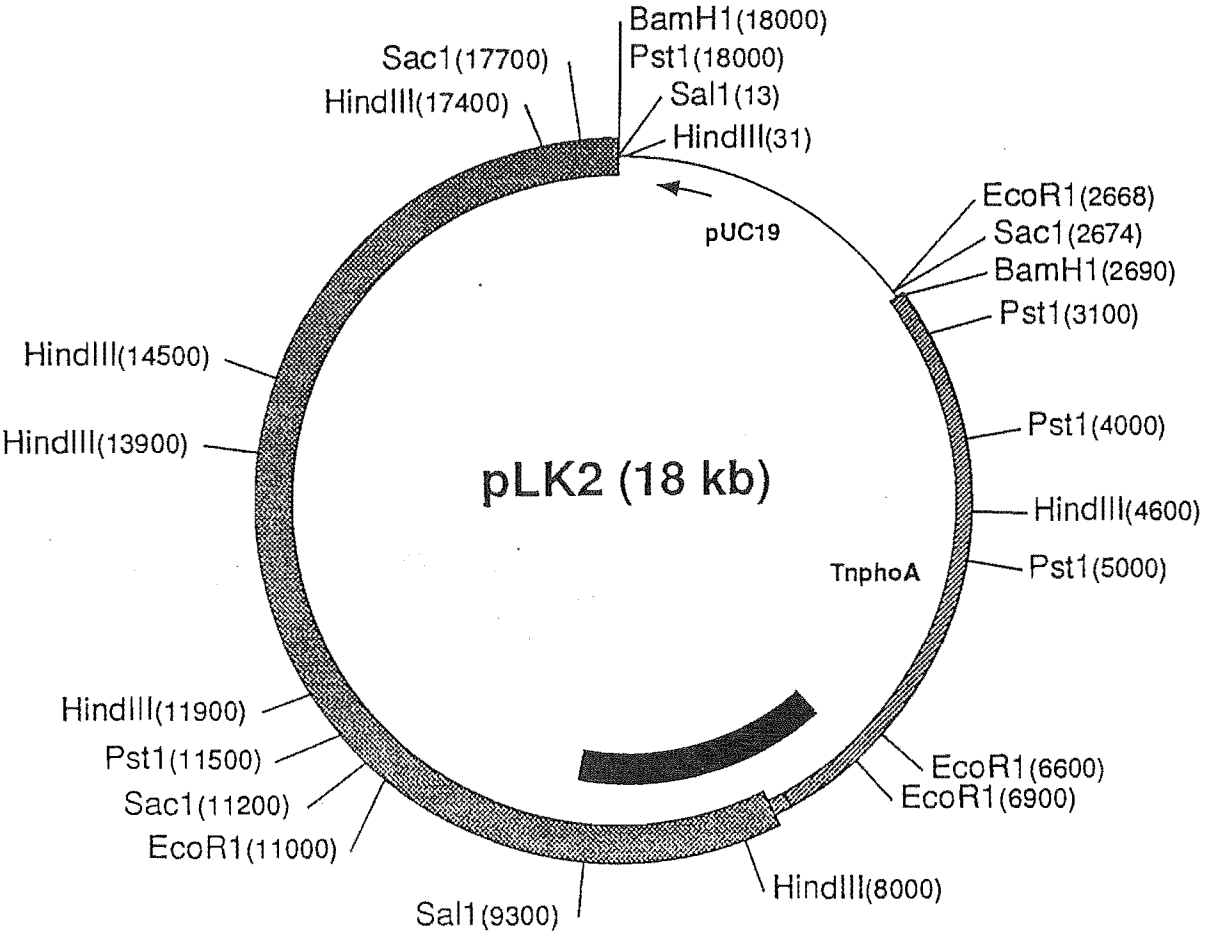
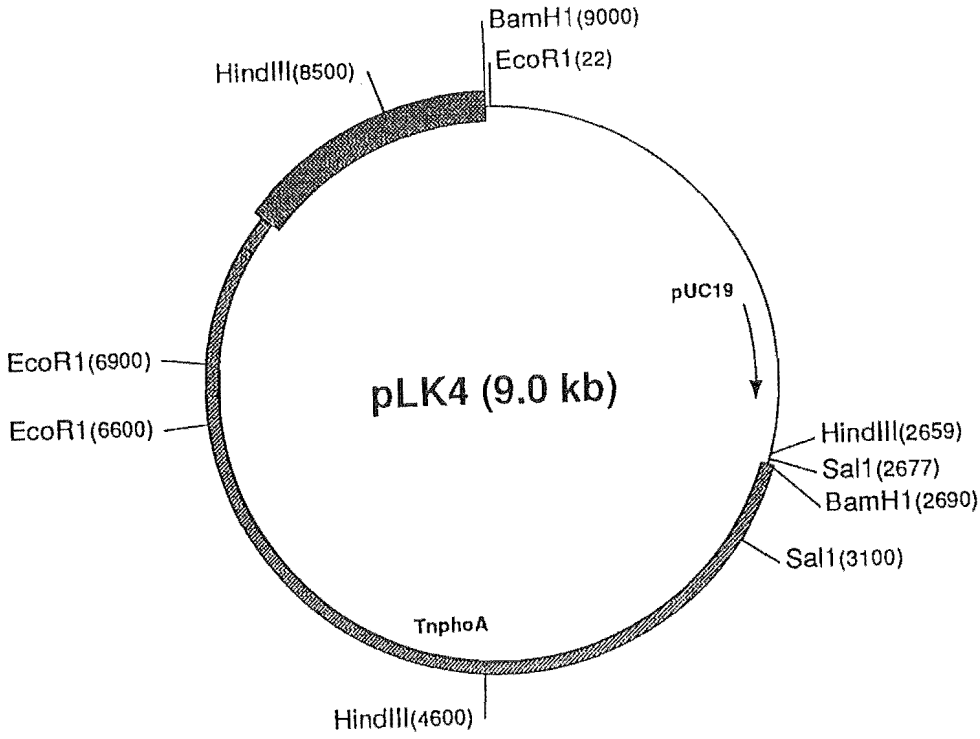


Figure 6.5: (Pages 74-76 incl.). Restriction maps of mutant fragment clones from Ant⁻ mutants with single Tn*phoA* insertions (pLK series). The 2.4 kb *Eco*R1-*Sal*1 fragment probe from pLK2, used for plasmid hybridisation (See Methods and Materials), is shown in bold. **pLK2 is derived from mutant EhA17g.**

——— pUC19

▭ Tn*phoA*

▭ Eh1087 mutant fragment



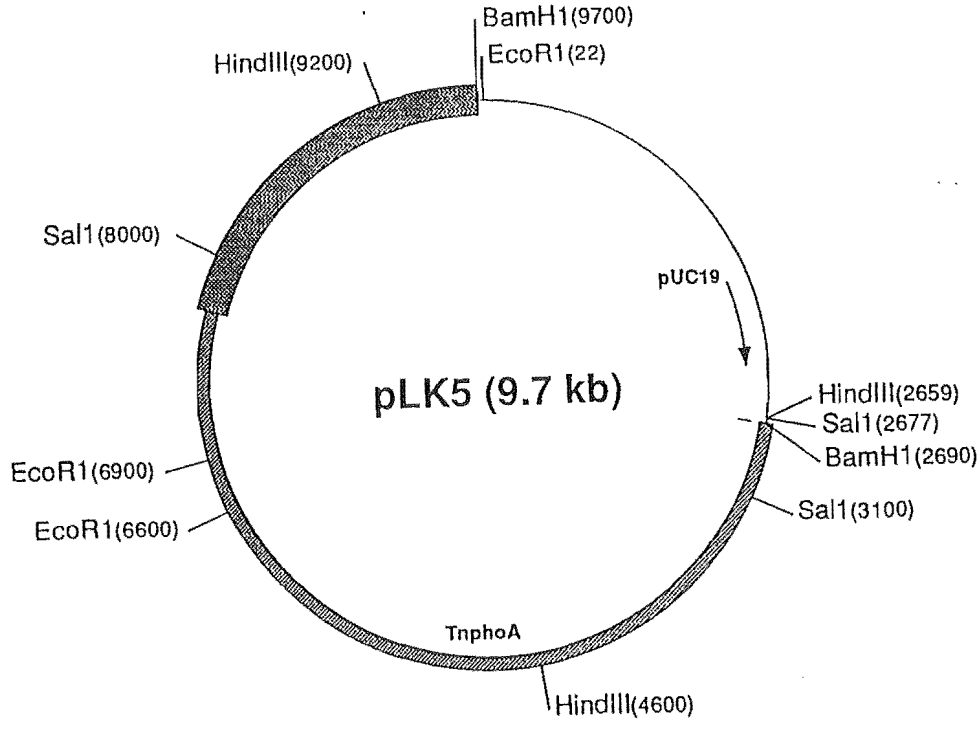
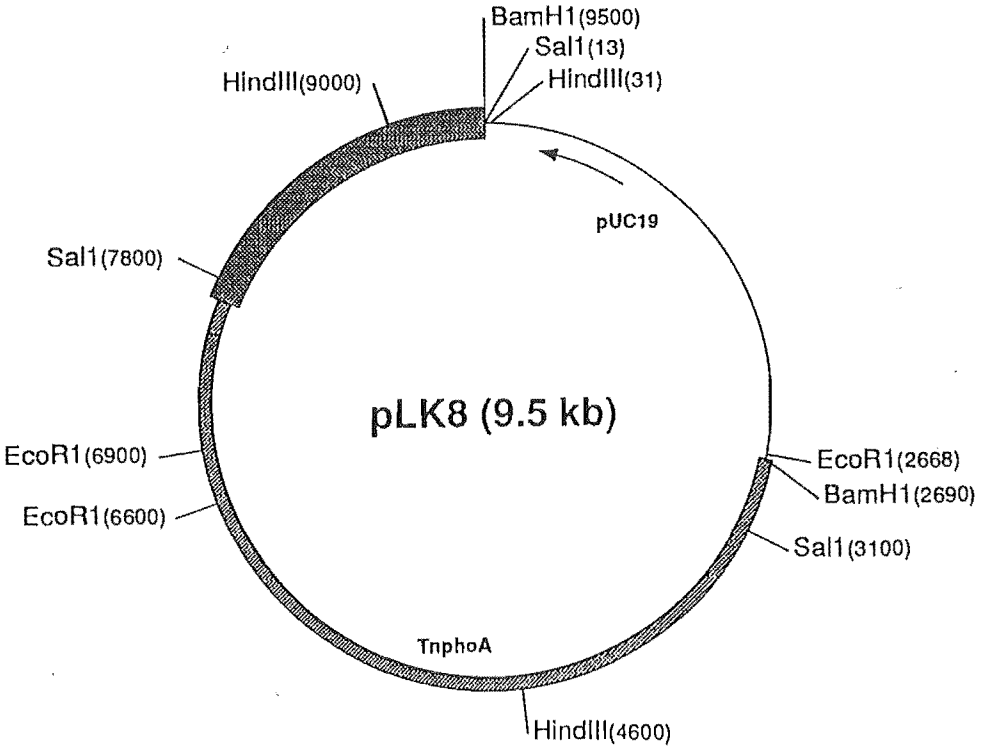


Figure 6.5: (cont.)
pLK4 and pLK5 are derived from mutant EhA19f and EhA11g, respectively.



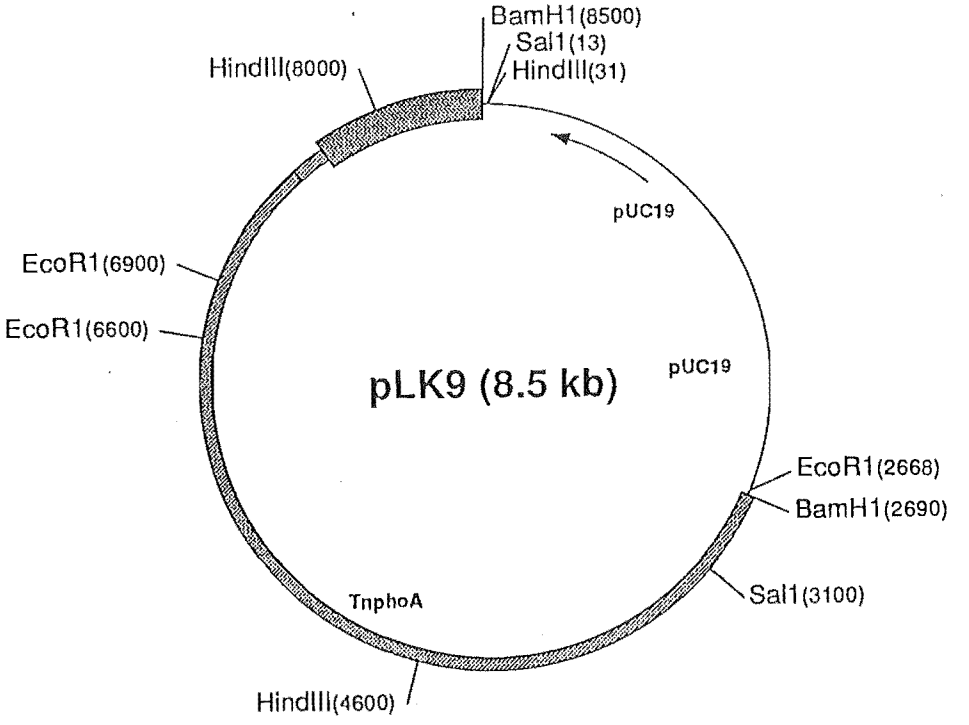


Figure 6.5: (cont.)
pLK8 and pLK9 are derived from mutant EhA46a and EhA12e, respectively.

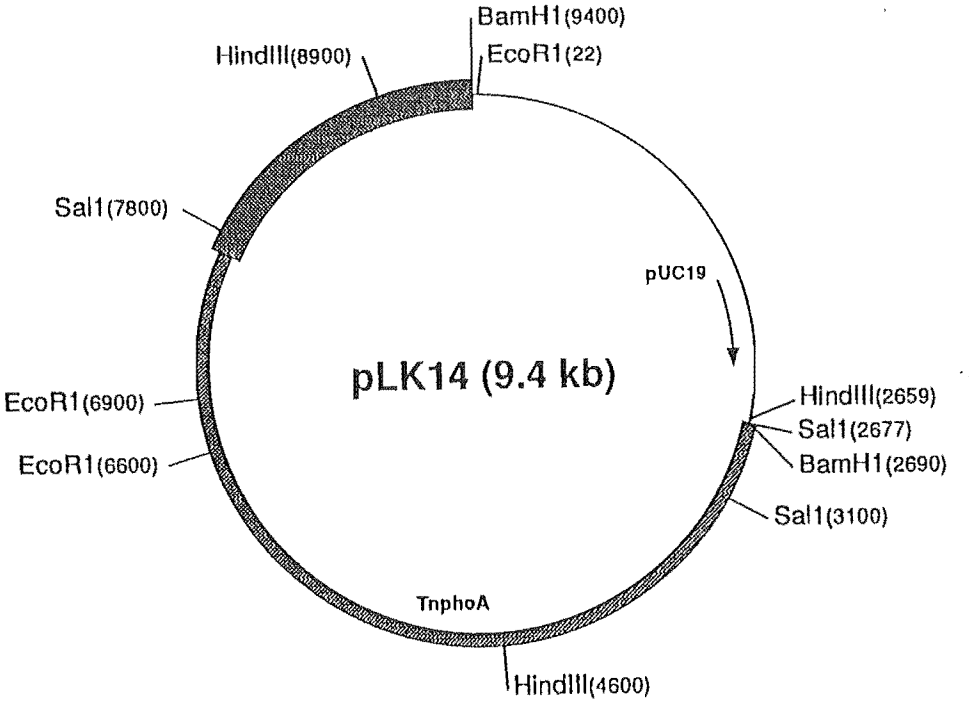


Figure 6.6: Restriction map of mutant fragment clone pLK14, from Ant$^-$, phoA$^+$ mutant EhA21a.

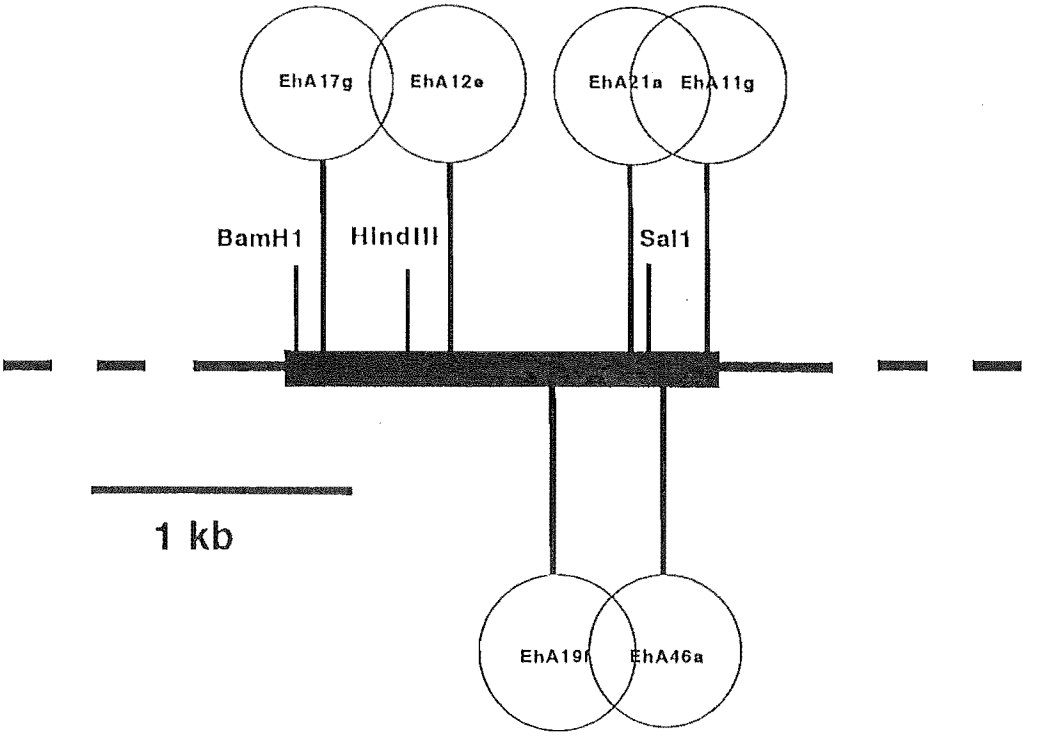


Figure 6.7: The Tn*phoA*-insertion site for each Ant$^-$ mutant is indicated by a paddle symbol, labelled with the corresponding mutant designation. Tn*phoA* insertions in the Ant$^-$ mutants mapped within a 1.5 kb region of Eh1087 DNA.

Eh1087 carries a large indigenous plasmid approximately 200 kb in size (Figure 6.8). A Southern blot of a plasmid visualisation gel was hybridised with a 2.4 kb DNA fragment probe prepared from mutant fragment clone pLK2. The probe hybridised to the plasmid band (Figure 6.8), indicating that the mutations in antibiotic activity were plasmid-borne, not chromosmal.

DISCUSSION

In order for transposon mutagenesis to be successful, an appropriate transposon delivery system must be employed. The delivery systems used in this study were designed for application to a wide range of gram negative bacteria (149). However, co-maintenance of the incompatible plasmids, pRT291 and pPH1JI, in Eh1087 recipients indicated that this plasmid delivery system may be unsuitable for *E. herbicola*.

Other commonly used transposon delivery systems have also been reported to be inappropriate for *Erwinia* species. One example is the suicide vector plasmid, pJB4J1, a derivative of pPH1JI, carrying Mu sequences and inserted Tn*5* (18). The presence of Mu sequences in this plasmid vector apparently makes it unstable, resulting in plasmid suicide and Tn*5* transposition (18). However, survival of this plasmid has been reported in *E. amylovora* (144) and *E. carotovora* (186). In *E. carotovora*, survival of pJB4J1 was found to be highly strain dependent (186).

Lambda-mediated transposon mutagenesis has also been used in *Erwinia* species (45, 132, 144, 163). Use of λ requires that *Erwinia* species are first made sensitive to λ infection by introduction of the *lamB* gene (45, 132). However, the frequency of transduction was found to vary significantly between λ-sensitive *Erwinia* strains (45, 132).

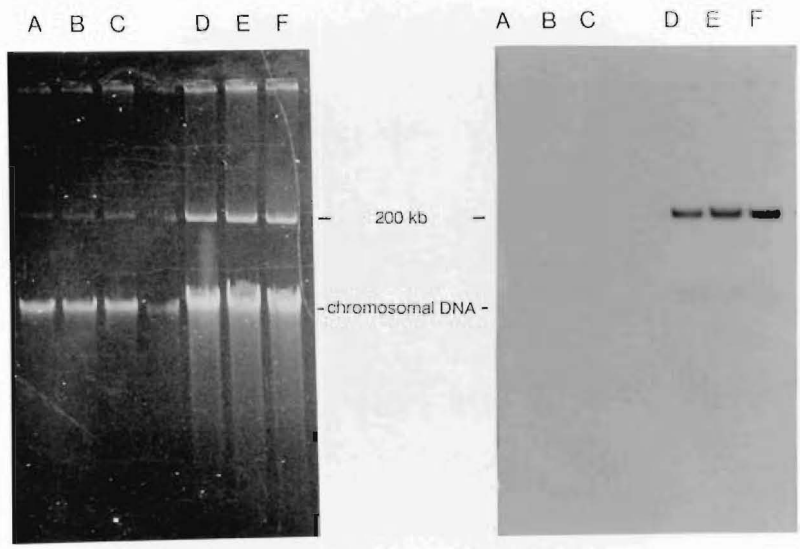


Figure 6.8: Agarose gel elctrophoresis of plasmid DNA from Eh1087 and Southern blot hybridisation with a pLK2 fragment probe. Lanes A), B) and C) *Agrobacterium tumefaciens* plasmid marker. (The >300 kb plasmid of *A. tumefaciens* is not visible on this gel). Lanes D), E) and F) Eh1087. (Overflow of the Eh1087 sample to the left of lane D can be seen).

The results of this study, and those of other researchers, highlight the problems that can arise when applying established molecular techniques to bacterial species with undefined genetic backgrounds.

Tn*phoA* mutagenesis of Eh1087 was successful using the suicide vector plasmid, pRT733 (149). This plasmid is derived from pBR322 and carries the origin of replication of plasmid R6K and the mob region of pRP4 but is unable to replicate in the absence of the *λpir* gene product (110).

Molecular characterisation of mutants

Double Tn*phoA* insertions were observed for 4/12 Ant$^-$ mutants and transposition of IS50 sequences, separately from the transposon molecule, was observed for 2/12 Ant$^-$ mutants. Transposition of insertion sequences of Tn5 and other composite transposons is known to occur (17, 83) and Kleckner *et al.* (83) comment that the transposition of IS50 sequences of Tn*5* actually occurs at a much higher frequency than transposition of the complete transposon. Tn*5* mutagenesis studies of other *Erwinia* strains have also reported multiple Tn*5* insertions and IS insertions in a large proportion of mutants obtained (144, 163).

There are several indications that Tn*phoA* insertion in Eh1087 was not random. The lack of auxotrophic mutations, combined with the high rate of Ant$^-$ mutants (1.2×10^{-2}) obtained, suggest that hot spot insertions of Tn*phoA* may have occurred. Transposon insertion is not entirely random and the occurrence of hot spots for transposon insertion has been reported before (83, 132). Similarly high ratios of Tn*5*-induced mutants of *E. herbicola*, defective in bacteriocin production, have been reported elsewhere, although the possibilty that mutants obtained arose as progeny of a single mutation could not be discounted in this study (55).

In addition to the high rate of Ant$^-$ mutations arising from transposon mutagenesis, the tight clustering of Tn*phoA* insertion points within a 1.5 kb region of DNA for these mutants suggests non-random insertion. Antibiotic biosynthesis generally involves multi-enzyme pathways, the genes for which tend to be grouped in 20-30 kb regions of DNA (101). Therefore, it is expected that entirely random mutagenesis would generate Eh1087 Ant$^-$ mutations mapping over a larger region of DNA than was observed.

In one of the Ant$^-$ mutants obtained, Tn*phoA* insertion resulted in an active *phoA* gene fusion, as evidenced by blue colonies on medium containing XP. The restriction map of this insertion showed overlaps with the restriction maps of the other Ant$^-$ mutations obtained, suggesting that the mutated locus codes for a secreted product. This would indicate that the mutations obtained lie in a structural gene(s) for the antibiotic and not in a regulatory gene(s).

Proteinase K digestion did not inactivate the antibiotic activity in cell-free culture supernatants of Eh1087 (See Chapter 5), indicating that the antibiotic is non-peptide. It may be that the mutated gene codes for a secretory signal peptide which is involved in transport of the antibiotic across the cell membrane, but which is not present on the secreted antibiotic. Alternatively, the mutated gene may code for a peptide moiety that is not directly involved in antibacterial activity. Thus, proteolytic degradation of the secreted antibiotic would yield a non-peptide, β-lactam subunit(s) with biological activity.

It must be noted that because the Tn*phoA* insertion resulting in a *phoA* gene fusion came from a double insert mutant (EhA21a), restriction map comparison alone is inadequate to definitely ascertain that the gene fusion lies in the same locus as that of the other Ant$^-$ mutations obtained. Marker exchange of the Tn*phoA* gene fusion with wild type Eh1087 would be necesary to confirm that this Tn*phoA* insertion occurs at the same locus as that of the other Ant$^-$ mutants.

Eh1087 Ant$^-$ mutants lacked the yellow pigmentation of the wild type colonies, indicating a linkage between pigmentation and antibiotic production. Information exists on the genetic organisation of antibiotic/bacteriocin production in *E. herbicola* for two other strains (55, 163) and in neither of these strains is a linkage between pigmentation and antibiotic production observed. In *E. herbicola* Eh112Y yellow pigmentation and bacteriocin production are encoded on separate indigenous plasmids (27, 54, 55). In *E. herbicola* Eh252 antibiosis is chromosomally encoded (163).

Antibiotic production in Eh1087 is plasmid encoded on an indigenous plasmid approximately 200 kb in size. Numerous indigenous plasmids are present in *E. herbicola* (30, 54, 55, 87, 163) and *E. amylovora* (120, 137, 145). *E. herbicola* is capable of conjugative transfer of plasmids of the *E. coli* incompatiblity groups F and P-1, both intergenerically, with other enteric bacilli, and interspecifically, between other *Erwinia* species (28, 29, 56, 86). Plasmid transfer of RP1 from *E. coli* to *E. herbicola* and from *E. herbicola* to *E. amylovora*, has been demonstrated to occur *in planta* (86). Conjugational transfer of virulence determinants in *E. amylovora* has been reported (126). This evidence suggests that genes for secondary metabolites, such as pigments and bacterial toxins, that are located on indigenous plasmids, may be transferable between different *Erwinia* strains, such that indigenous plasmids form a reservoir of genetic information which is available to numerous epiphytic *Erwinia* strains in the natural environment.

Biological role of the antibiotic

Eh1087 Ant$^-$ mutants failed to inhibit Ea8862 in immature pear fruit and failed to produce an antibiotic activity on solid medium or in broth culture. The inability of Ant$^-$ mutant strains to inhibit *E. amylovora* was not due to impaired competitive

ability, resulting from a reduced growth rate, as growth rates in HSN broth and in immature pear fruit were similar for mutants and wild-type Eh1087. This indicates a role for antibiotic production by Eh1087 in the inhibition of *E. amylovora*.

IPFA results show that mutants of Eh1087 deficient in antibiotic production fail to show any protection from disease. This is in contrast to previous studies in which antibiotic-deficient mutants of inhibitory *E. herbicola* strains still show some degree of disease protection in plants (13, 163), suggesting that other factors, such as competition, also play a role in the inhibition of *E. amylovora* by *E. herbicola*. The work of Ishimaru *et al.* (74) also supports a role for competition in inhibition of *E. amylovora* by *E. herbicola*, as mutants of *E. amylovora* showing multiple resistance to the antibiotics of *E. herbicola* EhC9-1 are no less inhibited by this strain than mutants with single antibiotic resistance.

In reality, it is likely that competition is also involved in the inhibition of *E. amylovora* by Eh1087. It is possible that modifications to the plant bioassay system employed in this study could highlight a competition factor in inhibition by Eh1087. However, the conditions used (high bacterial populations) were comparable to those of Vanneste *et al.* (163), in which antibiotic-deficient mutants of Eh252 still showed significant inhibition of *E. amylovora*. This suggests that antibiosis by Eh1087 is relatively more important to the inhibitory potential of this strain than is the contribution made by antibiosis in other inhibitory *E. herbicola* strains.

CHAPTER 7

COMPLEMENTATION OF ANT⁻ MUTANTS OF EH1087

SUMMARY

A genomic library of Eh1087 was constructed in the cosmid vector pLAFR3. Cosmids containing cloned DNA homologous to the region of Tn*phoA* insertions in Ant⁻ mutants were selected by DNA hybridisation. Three positively hybridising cosmids complemented Ant⁻ mutants, restoring inhibitory activity against Ea8862 *in vitro* and in immature pear fruit.

pLAFR3 was found to be unstable in *E. herbicola* during prolonged laboratory culture, resulting in progressively weaker complementation by complementing cosmids.

An 8 kb cosmid fragment subclone, pAH8, restored weak inhibitory activity to Ant⁻ mutant EhA17g. This complementation was enhanced by the presence *in trans* of a 5 kb cosmid fragment subclone, pBE5B. These two cloned fragments mapped within a 17 kb region of cosmid cloned DNA.

INTRODUCTION

Antibiotic biosynthesis in micro-organisms usually involves multiple genes, often linked in gene clusters which may extend over 20 - 30 kb (101). In order to isolate and characterise genes of interest, genomic libraries can be created in bacteriophage or cosmid vectors. Cosmids are plasmids containing the λ*cos* site, which is recognised during packaging of DNA into phage heads and which enables

cloned DNA to be packaged *in vitro* for efficient transduction into *E. coli* (40). Use of cosmid vectors enables large DNA regions to be cloned, thereby facilitating the analysis of clusters of linked genes.

Once cosmids from a library have been identified that complement mutants back to the wild-type phenotype, further complementation analysis with subcloned restriction fragments from the cosmids can be used to more accurately identify the coding regions involved.

The cosmid vector pLAFR3 was designed as a broad host range vector, capable of being mobilised into a wide range of Gram negative bacteria (41). pLAFR3 has been reported to have variable stability *in planta* (90, 116).

In this study, cosmids complementing Ant$^-$ mutants of Eh1087 were isolated and restriction mapped and two fragments were subcloned, which complemented Ant$^-$ mutant EhA17g *in trans*.

METHODS AND MATERIALS

Bacterial strains and plasmids

Bacterial strains and plasmids used are shown in Table 7.1. Plasmid constructions are shown in Table 7.2.

Table 7.1: Bacterial strains and plasmids.

Strain or plasmid	Characteristics[a]	Reference or source
Erwinia amylovora		
Ea8862		See Table 6.1
Erwinia herbicola		
Eh1087	wild-type *E. herbicola*	" " "
EhA12e	Eh1087 Ant$^-$ mutant	" " "
EhA14b	" " "	" " "
EhA17g	" " "	" " "
EhA19f	" " "	" " "
EhA20g	" " "	" " "
EhA46a	" " "	" " "
Escherichia coli		
DH5α		" " "
HB101	*supE hsdS recA ara proA lacY galK rpsL xyl mtl*	Bolivar and Backman (23)
LE392	*supE supF hsdR galK galT metB trpR lacY*	Murray *et al.* (117)
pRK2013	Km^R Tra$^+$ helper plasmid pRK2 replicon	Ditta *et al.* (41)
pLAFR3	Tc^R mob$^+$ Tra$^-$ *LacZα* *λcos* pRK2 replicon	Stascawicz *et al.* (142)
pACYC184	Cm^R Tc^R p15A replicon	Chang and Cohen (26)
pBR322	Ap^R Tc^R pMB1 replicon	Bolivar *et al.* (24)

[a] Km^R, Tc^R, Cm^R and Ap^R indicate resistance to kanamycin, tetracycline, chloramphenicol and ampicillin, respectively.

Table 7.2: Plasmid constructions.

Plasmid	Vector	Insert description[a]
pLA215	pLAFR3	complementing Eh1087 library cosmid
pLA3255	"	" " " "
pLA3272	"	" " " "
pLA424	"	partially complementing " "
pLA2170	"	" " " "
pLA2305	"	" " " "
pAB1131	pACYC184	*Bam*H1 11 kb subclone of pLA3255 (NOL)[b]
pAB1143	"	*Bam*H1 11 kb subclone of pLA3255 (OL)
pAH8	"	*Hin*dIII 8 kb subclone of pLA3255 (OL)
pBH3.8	pBR322	*Hin*dIII 3.8 kb subclone of pLA3255 (OL)
pBH4.2	"	*Hin*dIII 4.2 kb subclone of pLA3255 (OL)
pBE5A	"	*Eco*R1 5 kb subclone of pLA3272 (OL)
pBE5B	"	*Eco*R1 5 kb subclone of pLA3272 (NOL)
pLE5A	pLAFR3	*Eco*R1 5 kb subclone of pLA3272 (OL)

[a] Position of frgment subclones from complementing cosmids, in relation to the overlapping region, are shown in Table 7.10.

[b] OL indicates the subcloned fragment lies within the overlapping region of the complementing cosmids and NOL indicates the subcloned fragment lies outside the overlapping region of the complementing cosmids

Enzymes, substrates and antibiotics

All restriction enzymes and buffer solutions were obtained from BRL. Enzymes were used at a concentration of 3-5 U.μg^{-1} DNA. Antibiotics and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (XG) were obtained from Sigma Chemical Co. and were used at the following concentrations: Rif 50 $\mu g.ml^{-1}$, Km 50 $\mu g.ml^{-1}$, Tc 15 $\mu g.ml^{-1}$, Cm 35 $\mu g.ml^{-1}$ and XG 40 $\mu g.ml^{-1}$. Bacterial alkaline phosphatase (BAP) and T4 DNA ligase were supplied and used as previously indicated (Chapter 6). Calf intestinal phosphatase (CIP) was supplied by Boehringer Mannheim and used according to Sambrook *et al.* (133). After dephosphorylation the enzyme was inactivated by heating to 75°C for 10 min in the presence of 5 mM EDTA pH8. Dephosphorylated DNA was phenol:chloroform extracted and ethanol precipitated to remove CIP prior to ligations. RNase (Sigma) was used at a concentration of 40 $\mu g.ml^{-1}$.

Construction of a genomic library of Eh1087

Eh1087 genomic DNA was purified by the method of Scott *et al.* (135) and partially digested with *Sau*3A. Partially digested DNA was size fractionated on a linear sucrose gradient to purify DNA fragments 25-30 kb in size. The fragment ends were dephosphorylated to prevent self-ligation. The 25-30 kb dephosphorylated DNA fragments served as insert DNA. Cosmid pLAFR3 individual vector arms were prepared using the method of Stascawicz *et al.* (142). Insert DNA was ligated to pLAFR3 vector arms and packaged into λ phage heads *in vitro*. Test and preparative libraries were made according to the method of Fleischmann *et al.* (50).

A) Preparation of Eh1087 Insert DNA

i). *Sau*3A partial digestion

In a series of pilot reactions, Eh1087 DNA (400 ng) was incubated with *Sau*3A (0.1 U.μg^{-1} DNA) for different periods of time to determine partial digest conditions giving the greatest concentration of DNA fragments 25-30 kb in length, according to the method of Fleischmann *et al.* (50). Aliquots from each digest were analysed on a 0.3% agarose gel at 1 V.cm^{-1} for 12-16 hours, with *Hin*dIII- and *Xho*1-digested λ DNA included as molecular weight markers. From the pilot reactions, an optimum incubation time of 7.5 min was chosen for *Sau*3A partial digestion.

Three scaled-up *Sau*3A digests (25 μg DNA each) were carried out with incubation times of 5, 7.5 and 10 min, respectively, and duplicating all other conditions of the pilot digests. The extent of digestion was analysed as before.

The DNA from the scaled up digests was pooled, gently extracted twice with phenol:chloroform, precipitated with two volumes of ice-cold ethanol and redissolved in TE prior to sucrose density gradient centrifugation.

ii). Sucrose density gradient centrifugation

*Sau*3A partial digest DNA fragments 25-30 kb in length were purified by sedimentation through a 10-40% (w/v) continuous sucrose gradient, using the method of Sambrook *et al.* (133), with the following modifications: 1) gradients were centrifuged in a SW27 rotor for 24 hours at 26,000 rpm at 15°C, 2) bulk digest DNA was not heated to 65°C prior to loading onto the gradient, 3) fractions (0.5 ml) were withdrawn from the top of the gradient and aliquots (10 μl) of every second fraction were analysed by electrophoresis through 0.4% agarose at 1V.cm^{-1}, with *Hin*dIII- and *Xho*1-digested λ DNA, adjusted to 20% sucrose, included as molecular weight standards and 4) following electrophoresis, pooled gradient fractions were diluted three-fold in TE, adjusted to a concentration of 2M ammonium acetate and ethanol precipitated.

iii). Dephosphorylation of DNA

Purified DNA fragments (25-30 kb) from sucrose density gradient centrifugation were dephosphorylated with BAP for one hour at 65°C (20 U.μg^{-1} DNA) by the method of Sambrook *et al.* (133), extracted twice with phenol:chloroform, ethanol precipitated and resuspended in TE to a final concentration of 600 ng.μl^{-1}.

B) Preparation of pLAFR3 Vector Arms

Cosmid pLAFR3 DNA was purified using CsCl density gradient purification according to the method of Sambrook *et al.* (133). Individual vector arms were prepared using the method of Stascawicz *et al.* (142). Briefly, purified pLAFR3 (20 μg) was digested with either *Hin*dIII or *Eco*R1. Completely linearised DNA was phenol extracted, ethanol precipitated and resuspended in TE. Individual vector arms were dephosphorylated with BAP, re-extracted with phenol:chloroform and ethanol precipitated as before.

To check the efficiency of linearisation and dephosphorylation of the vector arms each individual arm was self-ligated and transformed into *E. coli* DH5α. Separate ligations of each vector arm, containing 250 ng vector DNA and 2.5 U T4 DNA ligase in a final volume of 20 μl ligase buffer, were carried out. A control ligation containing *Bam*H1-digested, non-dephosphorylated pLAFR3 DNA was included. Ligations were incubated 3 hours at 20°C. 1, 5 and 10 μl volumes of each ligation were used to transform competent DH5α. Transformed DH5α colonies were selected on LB agar + Tc. (See Chapter 6 for method of transformation).

Individual dephosphorylated pLAFR3 arms were digested with *Bam*H1 to prepare ligatable vector arms. The ability of *Bam*H1-digested arms to re-ligate together was tested, as follows: *Eco*R1- and *Hin*dIII-prepared vector arms were incubated together (100 ng of each) with 2.5 U T4 DNA ligase in a final volume of 10 μl ligase buffer. The ligated DNA was electrophoresed through 0.4% agarose at 1 $V.cm^{-1}$ for 12-16 hours. Non-ligated pLAFR3 vector DNA was run as a standard.

C) Ligation of Insert DNA with pLAFR3 Vector Arms

A test ligation of *Sau*3A-digested insert DNA (300 ng) and vector arms (450 ng of each arm) was carried out, using the following conditions: Vector DNA:insert DNA mass ratio 3:1, total DNA concentration 170 $ng.\mu l^{-1}$ and T4 DNA ligase at 4 $U.\mu g^{-1}$. The ligations were incubated 3 hours at 20°C. The test ligation was packaged *in vitro* and a test library prepared, using the methods outlined below. For the final library, DNA was pooled from three ligations identical to the test ligation. Aliquots from each ligation were analysed by electrophoresis through 0.4% agarose at 1 $V.cm^{-1}$ for 12-16 hours, using pLAFR3 and λ DNA as molecular weight standards.

D) *In Vitro* Packaging and Infection of *E. coli* DH5α

Cosmid DNA was packaged *in vitro* into λ phage heads using Packagene packaging extract (Promega) according to the manufacturers instructions.

Test and preparative libraries were prepared according to the method of Fleischmann *et al.* (50). Dilutions (1:10 and 1:100) of the packaging mix were used to transduce DH5α, made susceptible to phage infection by overnight culture in LB broth containing 0.2% maltose. Cells were resuspended in 0.5 volume 10 mM $MgSO_4$ prior to infection. After phage adsorption (15 min at 37°C), LB broth (0.9 ml) was added and the cells incubated a further 60 min, to allow expression of the acquired antibiotic resistance marker. Cells were concentrated to 100 μl by centrifugation and plated out on LB agar, supplemented with Tc and XG, to select for cells containing pLAFR3 cosmids carrying inserted DNA. Optimal conditions for packaging and transduction of the final library were determined using the test library titration results.

To determine the number of colonies, N, necessary for construction of a complete library, the following formula was used: $N = In(1-P)/In(1-f)$, where P (0.99) is the probability of any one DNA sequence being represented in the library and f is the fractional proportion of the genome represented in any single cosmid. Based on the size of the *E. coli* chromosome, f was calculated as $2.5x10^4$ bp/$4x10^6$ bp.

The final library was constructed as above, using DNA from the pooled ligations. To harvest the library, DH5α colonies containing pLAFR3 cosmids carrying insert DNA (Tc^R, white) were re-picked onto fresh medium and re-incubated overnight. Colonies were carefully washed from the surface of the plates with PBS (NaCl 8g.l^{-1}, KCl 0.2 g.l^{-1}, Na_2HPO_4 1.44 g.l^{-1}, KH_2PO_4 0.24 g.l^{-1}, pH 7.4) The pooled bacteria were diluted 1:1 with 2X Hogness freezing solution (NaCl 3.0 g.l^{-1}, K_2HPO_4 12.6 g.l^{-1}, KH_2PO_4 1.8 g.l^{-1}, glycerol 35 ml.l^{-1}) and pelleted at 3,500 rpm, 10 min at 4°C. Cells were resuspended in 50 ml 1X Hogness freezing solution and 1 ml aliquots were stored frozen at -80°C.

Library screening

A) Colony Blots

Single colonies from the library were spotted onto LB+Tc plates overlaid with pre-cut Hybond N+ membrane filters and incubated 12-16 hours at 37°C. Filters were then removed from the plates and washed with 10% SDS for 3 minutes, followed by 0.5 M NaOH, 1.5 M NaCl for 5 minutes to fix liberated DNA onto the membranes. Finally, filters were neutralised with two washes in 0.5 M Tris, pH 8.0, 1.5 M NaCl and air dried. (Filter washings were carried out by placing membranes on top of Whatman No. 1 paper pre-soaked in the appropriate solution).

B) Preparation of Probe

A 7 kb *Eco*R1-*Sal*1 fragment from the plasmid pLK2 (Figure 7.1), was purified by electro-elution into DEAE cellulose paper as follows: DNA was electrophoresed in a thick 1% agarose gel + ethidium bromide (50 $\mu g.ml^{-1}$) with shallow wells (ie. wells only about half the thickness of the gel). The gel was slit just in front of the desired band, while visualising the DNA with long-range UV light. Prepared DEAE paper, cut just wider than the sample wells in the gel, was inserted into the slit and the gel was run at 200V until the DNA had run entirely into the DEAE paper. The paper was removed, quickly rinsed in TE and placed into an eppendorf tube containing 0.4 ml 1M NaCl + 0.05M arginine. The DNA was eluted from the paper by incubating at 70°C for at least one hour. The eluted DNA was ethanol precipitated prior to labelling. (DEAE paper was prepared for use by washing in 10 mM EDTA pH 8 for 10 min, then in 0.5M NaOH for 5 min and finally rinsed several times in distilled water. DEAE paper could be stored in distilled water at 4°C for several weeks).

The fragment DNA was ^{32}P-labelled using the BRL random primers labelling kit, according to the manufacturers instructions.

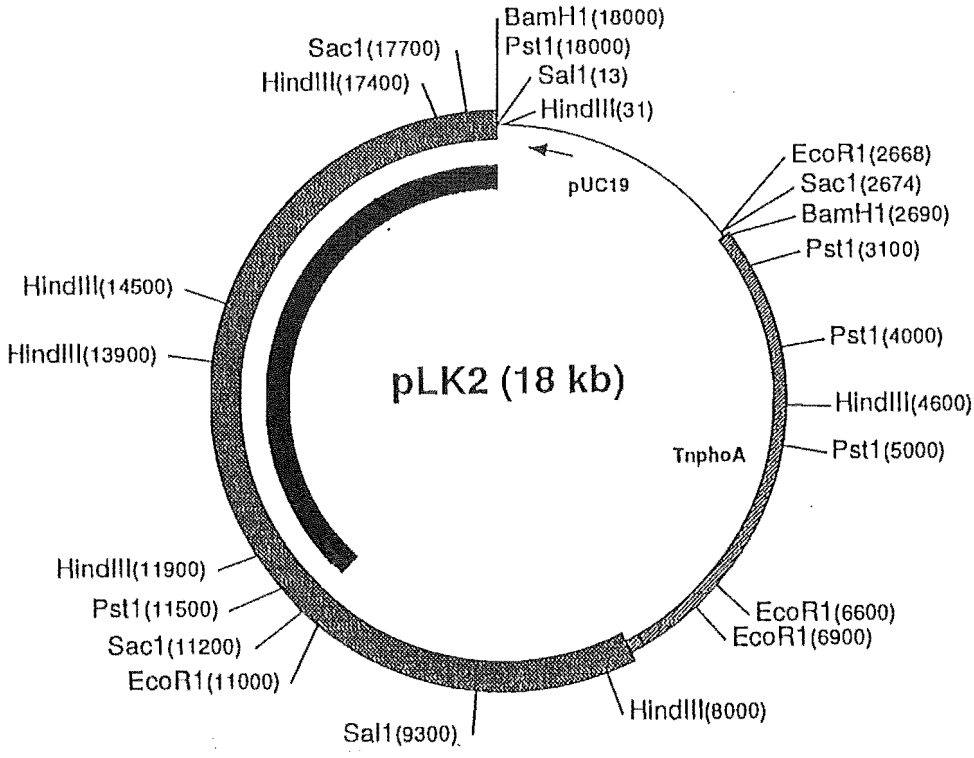


Figure 7.1: The 7 kb *Eco*R1-*Sal*1 fragment probe from pLK2 used to screen the Eh1087 genomic library is shown in bold.

C) Colony Hybridisation

Filters were sealed into plastic bags and pre-washed with gentle agitation for two hours in 3x SSC, 0.1% SDS at 65°C to remove cellular debris on the membrane surfaces. Filters were then pre-hybridised 2 hours in pre-hybridisation solution at 65°C before adding ^{32}P-ATP labelled DNA probe to give approximately 30,000 cpm.filter^{-1}. Pre-hybridisation solution was 2x SSC, 5x Denhardts solution, 0.126 mg.ml^{-1} sheared salmon sperm DNA (sssDNA), made from stock solutions of 20x SSC (3M NaCl, 0.3M Na_3citrate), 50x Denhardts solution (1% (w/v) bovine serine albumin, 1% (w/v) Ficoll^{TM} (Pharmacia), 1% (w/v) polyvinylpyrolidone) and 10 mg.ml^{-1} sssDNA. After addition of labelled probe, filters were incubated 16 hours at 65°C with gentle agitation. After DNA hybridisation was completed unbound DNA was removed from the filters by washing four times in 2x SSC, 0.1%

SDS at room temperature and once in 0.1x SSC, 0.1% SDS at 65°C for 15-45 minutes, until monitored radioactivity was reduced to an acceptable level.

Washed filters were dried, wrapped in gladwrap and autoradiographed at -80°C as before (Chapter 6).

Cosmid complementation of Ant⁻ mutants

pLAFR3 cosmid clones that positively hybridised to the mutant fragment probe, were mobilised from *E.coli* DH5α to Eh1087 Tn*phoA*-insertion mutants by tri-parental matings, using the helper plasmid, pRK2013 (41). Equal volumes (0.5 ml) of overnight LB broth cultures of donor (DH5α library strain, Tc^R), recipient (Eh1087 Tn*phoA*-insertion mutant, Rif^R, Km^R) and helper (containing pRK2013, Km^R) strains were mixed and matings carried out as for conjugative Tn*phoA* mutagenesis of Eh1087 (See Chapter 6). Exconjugant Eh1087 mutant strains carrying cosmids were selected on LB agar supplemented with Rif, Km and Tc.

Approximately 30 colonies from each tri-parental mating were tested for complementation to the Ant^+ phenotype by inoculation onto an HSN soft agar overlay of Ea8862 (See Chapter 3 for method). Those clones which produced zones of inhibition on overnight incubation were also assayed for inhibition of Ea8862 on immature pear fruit (See Chapter 3 for method) and for production of antibiotic activity in cell-free culture supernatants (See Chapter 5 for method).

To test for recombination in Eh1087 in the absence of antibiotic selection pressure, broth cultures (24 hour) of cosmid-carrying Eh1087 mutant strains, EhA17g(pLA215) and EhA17g(pLA3255), were grown in LB broth + Rif at 30°C. Single colonies from broth cultures were obtained by dilution plating. 500 colonies of each strain were plated onto a series of selective media to test for the loss of Km^R (indicating loss of inserted Tn*phoA*), loss of Tc^R (indicating loss of cosmid) and loss of both Km^R and Tc^R (indicating homologous recombination).

In addition, 500 colonies of each of the two cosmid-carrying strains were tested for recombination by plating as above after overnight incubation on HSN agar to test for the possibility of recombination occurring during *in vitro* complementation assays.

Cosmid restriction mapping

Restriction mapping, Southern blotting and hybridisation of cosmid DNA with a 2.4 kb *Eco*R1-*Sal*1 DNA fragment probe, derived from pLK2 (Figure 6.5), were carried out as previously described (Chapter 6). Cosmid DNA was purified using the following method: Cells from overnight LB broth + Tc culture (100 ml) of cosmid-carrying strains were sedimented by centrifugation at 12,000 rpm for 5 min at 4°C and the pellet resuspended in 10 ml solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8). Cells were lysed by the addition of 20 ml freshly prepared solution II (0.2N NaOH, 1% SDS) and incubation at 37°C for 15 min. Chromosomal DNA, released by cell lysis, was precipitated by the addition of 15 ml solution III (3M Na_3acetate pH 4.8) and storage on ice for 10 min. Lysed cells were centrifuged at 12,000 rpm for 10 min at 4°C and the pellet discarded. Cosmid DNA, remaining in the supernatant, was precipitated by the addition of 0.6 volume isopropanol (15 min at -20°C). The precipitated DNA was collected by centrifugation and resuspended in 2 ml TE. RNA was selectively precipitated by the addition of 2 ml cold 5M LiCl (5 min on ice) and the RNA pellet was collected by centrifugation at 8,000 rpm for 10 min at 4°C. Cosmid DNA was then ethanol precipitated. The pellet was resuspended in 500 μl TE and treated with RNAse (15 min at 37°C) to remove any remaining RNA. DNA was phenol:chloroform extracted and ethanol precipitated for final resuspension in 100 μl TE.

Subcloning of cosmid restriction fragments

Restriction fragments from the overlapping region of the complementing cosmids (and two fragments from the non-overlapping region) were subcloned (Table 7.2) into plasmids pACYC184 (*Bam*H1 and *Hin*dIII cloning sites) and pBR322 (*Eco*R1 and *Hin*dIII cloning sites) and into pLAFR3 (*Eco*R1 cloning site) as described below.

Restriction fragments 5.0 kb in length or smaller were purified from agarose gels by the method of Heery *et al.* (67) and cloned into linearised, dephosphorylated vectors, as described previously (Chapter 6), with the exception that dephosphorylation was carried out using CIP, not BAP.

Plasmid pLE5A was constructed by cloning the 5 kb *Eco*R1 fragment insert of pBE5A into pLAFR3, using a shotgun method, as previously described (Chapter 6). Prior to transformation of DH5α, the total ligated DNA was digested with *Cla*1 to linearise any self-ligated pBR322 (thus preventing transformation of DH5α with self-ligated pBR322). To check *Cla*1 digestion was effective, single colony lysis of Tc^R transformants was carried out to confirm presence of the pLAFR3 clone and absence of pBR322.

Plasmids pAB1131 and pAB1143 were constructed by cloning the respective 11 kb *Bam*H1 fragments directly from the cosmid into pACYC184, using shotgun cloning as above. Utilising *Bam*H1-insertional inactivation of the Tc^R gene of pACYC184, Cm^R Tc^S transformants were selected.

To construct the recombinant plasmid pAH8, *Hin*dIII 3.8 kb and 4.2 kb fragments from the cosmid overlapping region were first separately cloned into pBR322 to create pBH3.8 and pBH4.2, respectively. Using shotgun cloning, the *Hin*dIII fragment inserts of pBH3.8 and pBH4.2 were then cloned contiguously into pACYC184. Because pACYC184 is subject to insertional inactivation of its Tc^R gene, pACYC184 clones carrying inserted pBR322 (Tc^R) were eliminated by

counter selection of Cm^R transformants for sensitivity to Tc. ^{32}P-labelled DNA probes were prepared from the two *Hin*dIII fragments as previously described (Chapter 6) and 350 Cm^R, Tc^S transformants were separately hybridised with each of the probes using methods described previously (Chapter 6). Those transformants that hybridised to both of the *Hin*dIII fragment probes, indicating insertion of both fragments within the pACYC184 molecule, were selected for plasmid restriction mapping to determine the orientation of the restriction fragments.

DNA mini-preparations were made of all the constructed plasmid clones by the method of Sambrook *et al.* (133) and DNA was restriction mapped to confirm that the appropriate fragment had been cloned and to define the orientation of the inserted fragments.

Single colony lysis of transformed DH5α

Transformants putatively containing pLE5A were lysed by a single colony lysis to confirm the presence of the plasmid and the absence of pBR322. The method used was a modification of that of Sambrook *et al.* (133). Single colonies were lysed in 25 μl lysis solution (50 mM NaOH, 0.5% SDS, 5 mM EDTA) in wells in a microtitre tray. Approximately half the cell mass of each colony was transferred from the plate to the wells and dispersed by stirring using a sterile toothpick. Once cell lysis was complete, 40 μl sample buffer (40 mM Tris-HCl pH8, 20 mM Na_3acetate, 2 mM EDTA, 18 mM NaCl, bromophenol blue) was added to each well and contents mixed. Approximately 25 μl of each sample was electrophoresed in 0.8% agarose at 60 mA for 75-90 min. DH5α transformed with self-ligated pBR322 and non transformed DH5α were included as controls.

Complementation of Ant⁻ mutants with cloned cosmid fragments

The Ant⁻ mutant EhA17g was transformed with cosmid fragment subclones by electroporation. Cells were prepared for electroporation as follows: LB broth (100 ml), supplemented with Rif and Km, was inoculated with 1 ml of overnight culture of EhA17g and grown at 30°C to $O.D._{600}$ = 0.5. The culture was chilled on ice for 10-15 min and centrifuged at 2,000 rpm for 10 min at 4°C. The pellet was washed three times; firstly in 100 ml ice-cold sterile distilled water, secondly in 50 ml ice-cold sterile distilled water and finally in 2 ml sterile 2% glycerol (w/v). After the third wash cells were resuspended in 300 μl sterile 2% glycerol (w/v) and 40 μl aliquots were stored frozen at -80°C.

Electroporation was carried out using a BioRad Gene Pulser according to the instruction manual. Aliquots (40 μl) of prepared EhA17g were electroporated with 500 ng DNA with a 4.5 mSec time pulse at 12.5 $V.cm^{-1}$ (0.2 cm cuvette). Outgrowth was for one hour at 30°C in 1 ml SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Electrotransformed EhA17g was selected by plating on LB agar, supplemented with the appropriate antibiotics.

Complementation on HSN agar was analysed as previously described (Chapter 3).

RESULTS

Construction of Eh1087 genomic library

Eh1087 genomic DNA was partially digested with *Sau*3A (Figure 7.2) and fragments 25-30 kb in length were purified by sucrose density gradient centrifugation (Figure 7.3). This DNA served as insert DNA for pLAFR3. The efficiency of linearisation and dephosphorylation of individual pLAFR3 vector arms was checked by ligation and transformation into DH5α (Table 7.3) and was found to be 99.1% for the *Hin*dIII-prepared arm and 96.5% for the

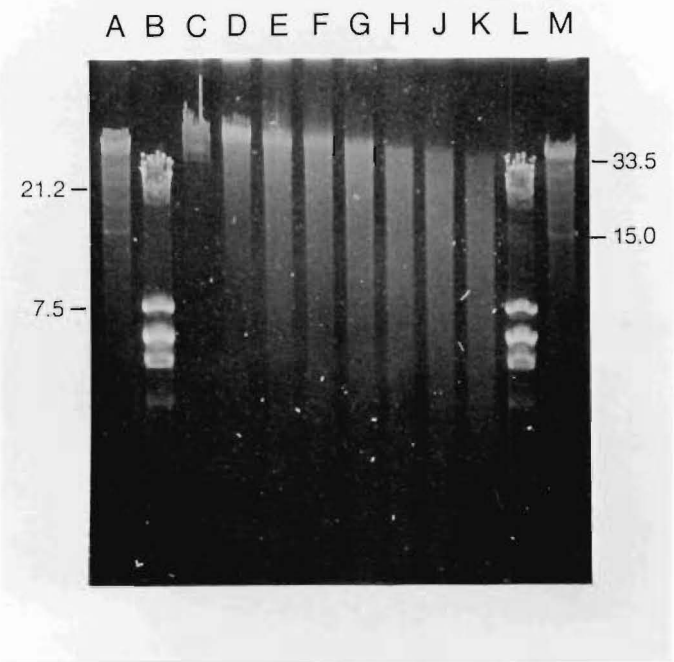


Figure 7.2: Time course of *Sau*3A digestion of Eh1087 genomic DNA. Lanes: A) *Xho*1-digested λ DNA, B) *Eco*R1-digested λ DNA, C) undigested λ DNA, D) *Sau*3A-digested Eh1087 DNA, t= 0 min, E) t=2.5 min, F) t= 5 min, G) t=10 min, H) t=15 min, J) t=20 min, K) t=30 min, L) *Eco*R1-digested λ DNA, M) *Xho*1-digested λ DNA.

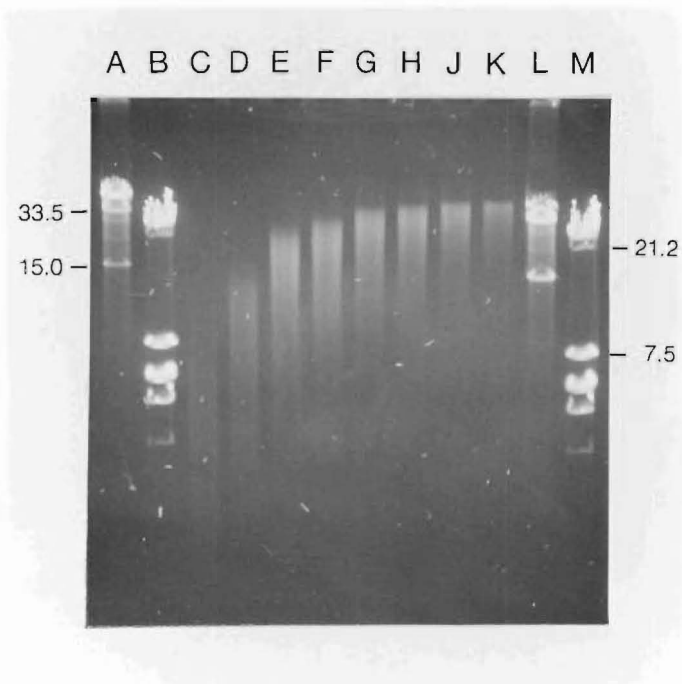


Figure 7.3: Sucrose density gradient centrifugation of a *Sau*3A partial digest of Eh1087 genomic DNA. Lanes: A) *Xho*1-digested λ DNA, B) *Eco*R1-digested λ DNA, C) - K) alternate sucrose gradient fractions, nos. 10 - 24, L) *Xho*1-digested λ DNA, M) *Eco*R1-digested λ DNA.

*Eco*R1-prepared arm. A test library was prepared and titred to 6 x 10^5 colonies per μg size-fractionated insert DNA (Table 7.4). Approximately 750 colonies were calculated to be necessary to ensure a 99% probability of every unique DNA sequence in the genome being represented in the library, based on the size of the *E. coli* chromosome. In total, 2200 colonies were stored as the library in order to ensure complete representation of genes.

Table 7.3: Efficiency of pLAFR3 vector arm preparation.

Transforming DNA (μl ligation reaction)	No. transformed DH5α (Tc^R)	Avg. preparation efficiency [100-(x/y)]
no DNA	nil	
pLAFR3 (y) 1 μl 5 μl	 240 510	
*Eco*R1 pLAFR3 arm (x) 1 μl 5 μl	 7 20	 96.5%
*Hin*dIII pLAFR3 arm (x) 1 μl 5 μl	 3 3	 99.1%

Table 7.4: Preparation of test library: Transformation of DH5α.

Treatment	No. transformed DH5α		
	blue	white	total
competent DH5α, 100 μl	nil	nil	nil
100 μl cells + 50 μl pkgd DNA	304	892	1196
100 μl cells + 50 μl 1:10 dil	48	163	211
100 μl cells + 50 μl 1:100 dil	2	10	12
200 μl cells + 100 μl pkgd DNA	600	1760	2360
200 μl cells + 100 μl 1:10 dil	74	312	386
200 μl cells + 100 μl 1:100 dil	8	27	35

When this library was screened, 11 positively hybridising cosmid clones were isolated. Of these, 3 cosmids, pLA215, pLA3255 and pLA3272, complemented the mutant EhA17g back to the Ant^+ phenotype on HSN agar (Figure 7.4) and in immature pear fruit (Table 7.5, Figure 7.5).

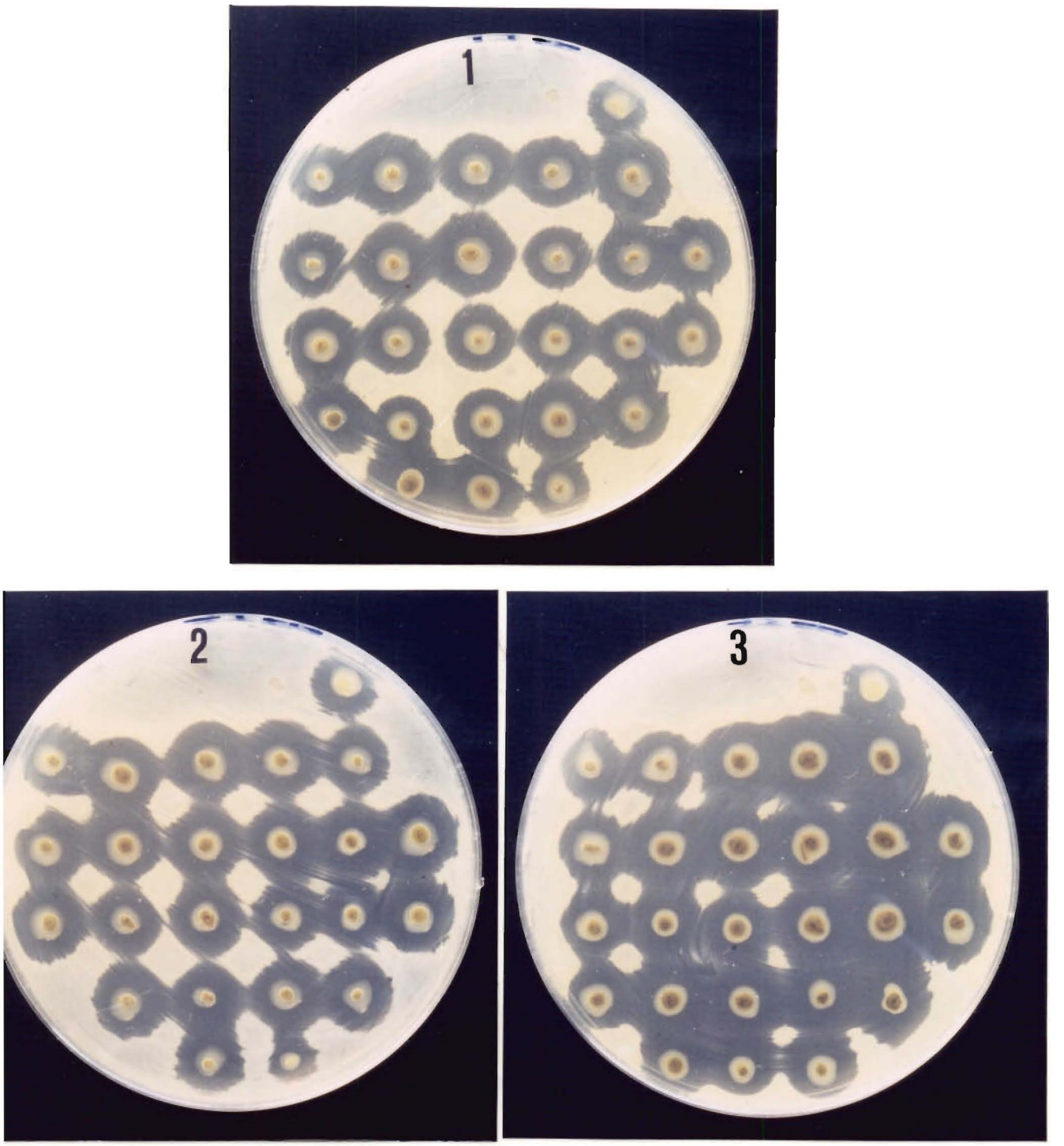


Figure 7.4: Complementation on HSN agar of Ant⁻ mutant EhA17g by cosmids from the Eh1087 genomic library. Plates: 1) EhA17g(pLA215), 2) EhA17g(pLA3255), 3) EhA17g(pLA3272). Controls on the top row of each plate, from left to right, are: *E. coli* DH5a, *E. coli* SM10(pRK2013), EhA17g and Eh1087.

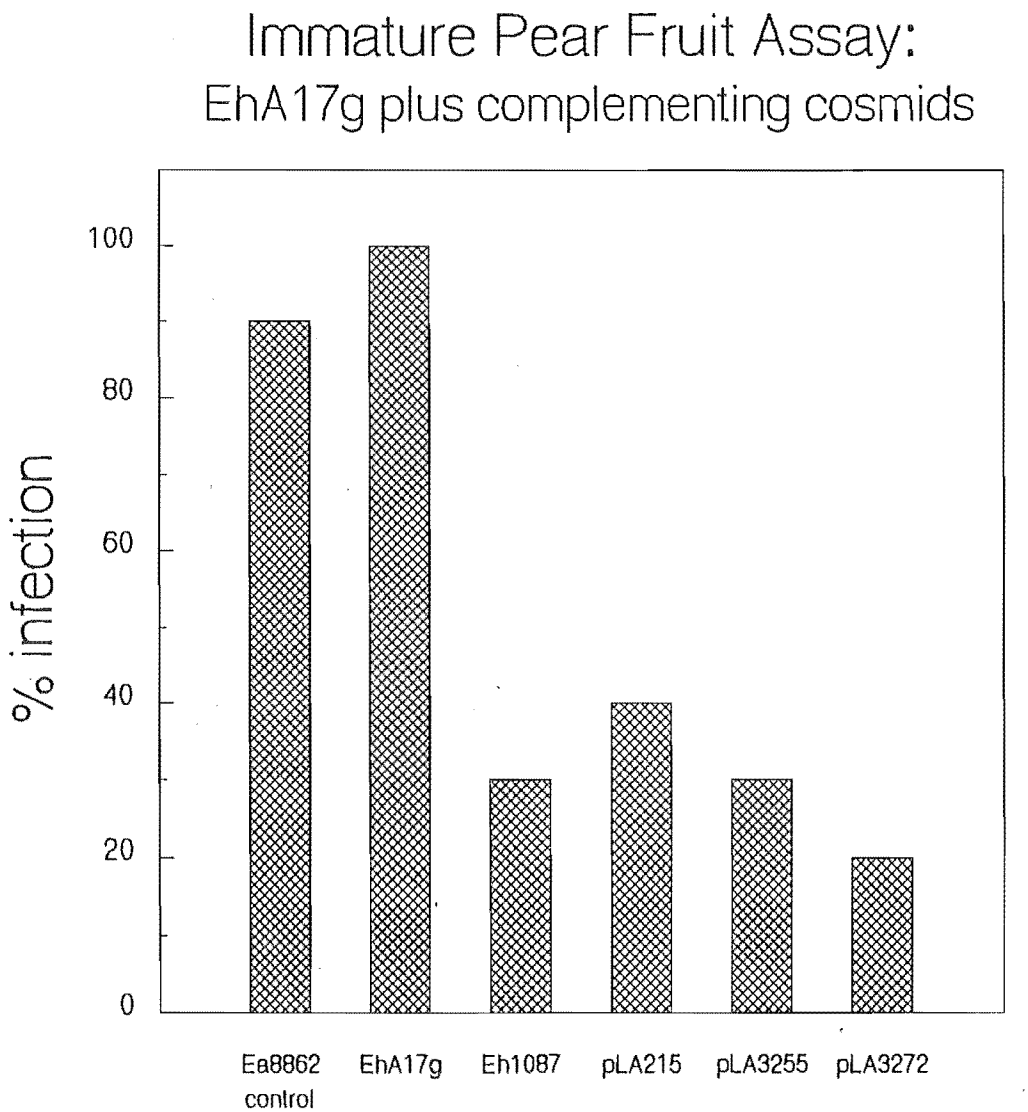


Figure 7.5: Complementation in immature pear fruit of Ant⁻ mutant EhA17g by cosmids from the Eh1087 genomic library. From left to right, % infection in pear fruit slices 3 days post inoculation is shown for Ea8862 alone and Ea8862 co-inoculated with EhA17g, Eh1087, EhA17g(pLA215), EhA17g(pLA3255) and EhA17g(pLA3272), respectively.

HSN culture supernatants of EhA17g(pLA215) and EhA17g(pLA3255) had faint antibiotic activity against Ea8862. EhA17g(pLA3272) did not show antibiotic activity in HSN supernatants. All the Ant$^-$ mutants in this study were complemented *in vitro* by these 3 cosmids (Table 7.6).

Table 7.5: Immature pear fruit assay of complementing cosmids.

Treatment	% Infection 3 days post inoculation		
	Assay 1	Assay 2	Assay 3
Saline control	0	0	0
Ea8862 control	100	90	90
Ea8862+Eh1087	50	30	10
Ea8862+EhA17g	100	100	100
Ea8862+EhA17g(pLA215)	80	40	10
Ea8862+EhA17g(pLA3255)	40	30	10
Ea8862+EhA17g(pLA3272)	60	0	10

Table 7.6: Complementation[a] of Ant$^-$ mutants by complementing cosmids.

Strain	pLA215	pLA3255	pLA3272
EhA11g	I	III	II
EhA12e	I	III	II
EhA17g	III	III	III
EhA19f	I	II	I
EhA20g	II	III	III
EhA46a	III	III	III

[a] I = >90% of colonies produce inhibition zones, all smaller than wild-type; II = 100% of colonies produce inhibition zones, of which 50-60% produce wild-type sized zones; III = 100% of colonies produce wild-type sized inhibition zones

The degree of restoration of Ea8862 inhibition by the complementing cosmids varied among the different Ant$^-$ mutants. For example, EhA19f was more weakly complemented overall than were the other Ant$^-$ mutants and EhA17g and EhA46a were more strongly complemented by all 3 cosmids. In addition, cosmid pLA215 complemented more weakly overall than pLA3255 or pLA3272.

Variability in the size of inhibition zones was also observed in the initial cosmid library screening, in which 3 "partially complementing" cosmids, pLA424, pLA2170 and pLA2305, restored *in vitro* inhibition of Ea8862 to mutant EhA17g, although inhibition zones were only half the size of those of wild-type Eh1087 and were only produced by appoximately half the colonies tested.

At the time of the initial screening of the cosmid library, the frequency of recombination in Ant$^-$ strains carrying homologous DNA inserts on cosmids was analysed. Conditions used were 24 hour broth culture (no added antibiotics) and overnight incubation on HSN agar. Results are shown in Table 7.7.

Table 7.7: Recombination in EhA17g cosmid-carrying strains in the absence of selection pressure: Analysis 1.

Strain	LB broth culture			HSN agar culture		
	Km^RTc^S	Tc^RKm^S	Tc^SKm^S	Km^RTc^S	Tc^RKm^S	Tc^SKm^S
EhA17g (pLA215)	75 (15%)	1 (0.2%)	2 (0.4%)	0	0	0
EhA17g (pLA3255)	82 (16%)	0	0	0	0	0

Number of colonies (500 tested) with the indicated antibiotic resistance are shown. Percentage figures are included in brackets.

In the absence of selection pressure (LB broth culture), recombination occurred with a low frequency (0 or 0.4%) in EhA17g carrying homologous DNA sequences on cosmids. The two Tc^SKm^S colonies of EhA17g(pLA215) that arose were Ant^+, indicating that homologous recombination had occurred. No loss of antibiotic resistance markers was observed after overnight incubation on HSN agar. pLAFR3 was lost from approximately 16% of cells after 24 hour LB broth culture, in the absence of selection pressure.

With repeated subculture over many months in the laboratory, complementation by cosmids appeared to become weaker or more variable, with inhibition zones being reduced in size and occasionally being absent, prompting a second recombination analysis to be carried out, nine months after the initial experiment. This analysis showed recombination occurring at high frequencies (Table 7.8).

Table 7.8: Recombination in EhA17g cosmid-carrying strains in the absence of selection pressure: Analysis 2.

Strain	HSN agar culture				
	Tc^RKm^R	[a] wTc^RKm^R	Tc^SKm^R	Tc^RKm^S	Tc^SKm^S
EhA17g (pLA3255)	139 (29%)	16 (3%)	0	0	345 (69%)
EhA17g (pLA3272)	63 (16%)	11 (3%)	14 (4%)	0	312 (78%)

[a] wTc^R indicates weakly Tc^R.
The number of colonies with indicated antibiotic resistance is shown. 500 colonies of EhA17g(pLA3255) were tested and 400 colonies of EhA17g(pLA3272) were tested. Percentage figures are included in brackets.

The inhibition phenotypes (ie. Ant^+ or Ant^-) of colonies showing different antibiotic resistance patterns were investigated (Table 7.9).

Table 7.9: Inhibition phenotype of EhA17g cosmid-carrying strains.

Strain	*In vitro* inhibition of Ea8862			Total
	Wild-type	Reduced	Absent	
EhA17g (pLA3255) Tc^RKm^R	30	0	0	30
EhA17g(pLA3272) Tc^RKm^R	30	0	0	30
EhA17g(pLA3255) wTc^RKm^R	9	7	0	16
EhA17g(pLA3272) wTc^RKm^R	5	6	0	11
EhA17g(pLA3272) Tc^SKm^R	7	0	3	10

Tc^SKm^S colonies had a wild-type Ant^+ phenotype, indicating homologous recombination between mutant and cosmid DNA had occurred, with concommittant loss of antibiotic resistance markers and restoration of wild-type DNA to the mutant. This recombination occurred with a high frequency (69% and 78%).

Colonies expressing a weak Tc^R showed a very reduced inhibition of Ea8862. These results suggested that pLAFR3 was increasingly unstable with time in laboratory culture in strains derived from Eh1087 and that progressive loss of complementing cosmids from mutants was responsible for the reduction in complementation observed.

Tc^SKm^R strains of EhA17g(pLA3272) arose that had both Ant^+ (7/10) and Ant^- (3/10) phenotypes. Loss of pLAFR3 could explain those colonies with an Ant^- phenotype. Restoration of the Ant^+ phenotype in Tc^SKm^R colonies could possibly be explained by the occurrence of a homologous recombination between cosmid and mutant DNA, downstream from the site of Tn*phoA* insertion, that cancelled out a polar effect exerted by the transposon.

Complementing cosmids pLA3255, pLA215 and pLA3272 and "partially complementing" cosmid, pLA424, were restriction mapped (Figure 7.6) and the overlapping region of cosmid DNA was determined by a combination of restriction map comparisons and DNA hybridisations of Southern blotted DNA digests with a mutant fragment probe derived from pLK2 (Figure 7.7a and b).

Cosmid restriction fragments from the overlapping region were subcloned (Table 7.2) and restriction mapped (Figure 7.8). These cosmid restriction fragments were analysed for *in vitro* complementation of mutant EhA17g (Table 7.10).

In an attempt to circumvent the need to re-introduce subcloned cosmid DNA back into the mutant *E. herbicola* background, the possibility of expression of the Eh1087 antibiotic in an *E. coli* background was investigated with *E. coli* strains HB101, DH5α and LE392. However, DH5α and HB101 failed to grow consistently on HSN medium and LE392 inhibited Ea8862 on the test medium. (*E. coli* was incubated overnight at 37°C and then inoculated with a soft agar overlay of Ea8862 and re-incubated at 30°C. Chloroform lysis of cells was not used as exposure to chloroform vapour inactivatd the Eh1087 antibiotic).

An 8 kb *Hin*dIII fragment subclone, pAH8, consisting of two smaller *Hin*dIII fragments from cosmid pLA3255, cloned contiguously into pACYC184, weakly complemented EhA17g *in vitro* (Table 7.10, Assay 4; Figure 7.9). This complementation was enhanced by the presence *in trans* of a 5 kb *Eco*R1 fragment subclone from pLA3272, pBE5B (Table 7.10, Assay 6; Figure 7.10). These two cosmid fragment subclones restored inhibitory activity to Ea8862 in immature pear fruit (Figure 7.11) and restored weak antibiotic activity in HSN broth culture supernatants (Results not shown).

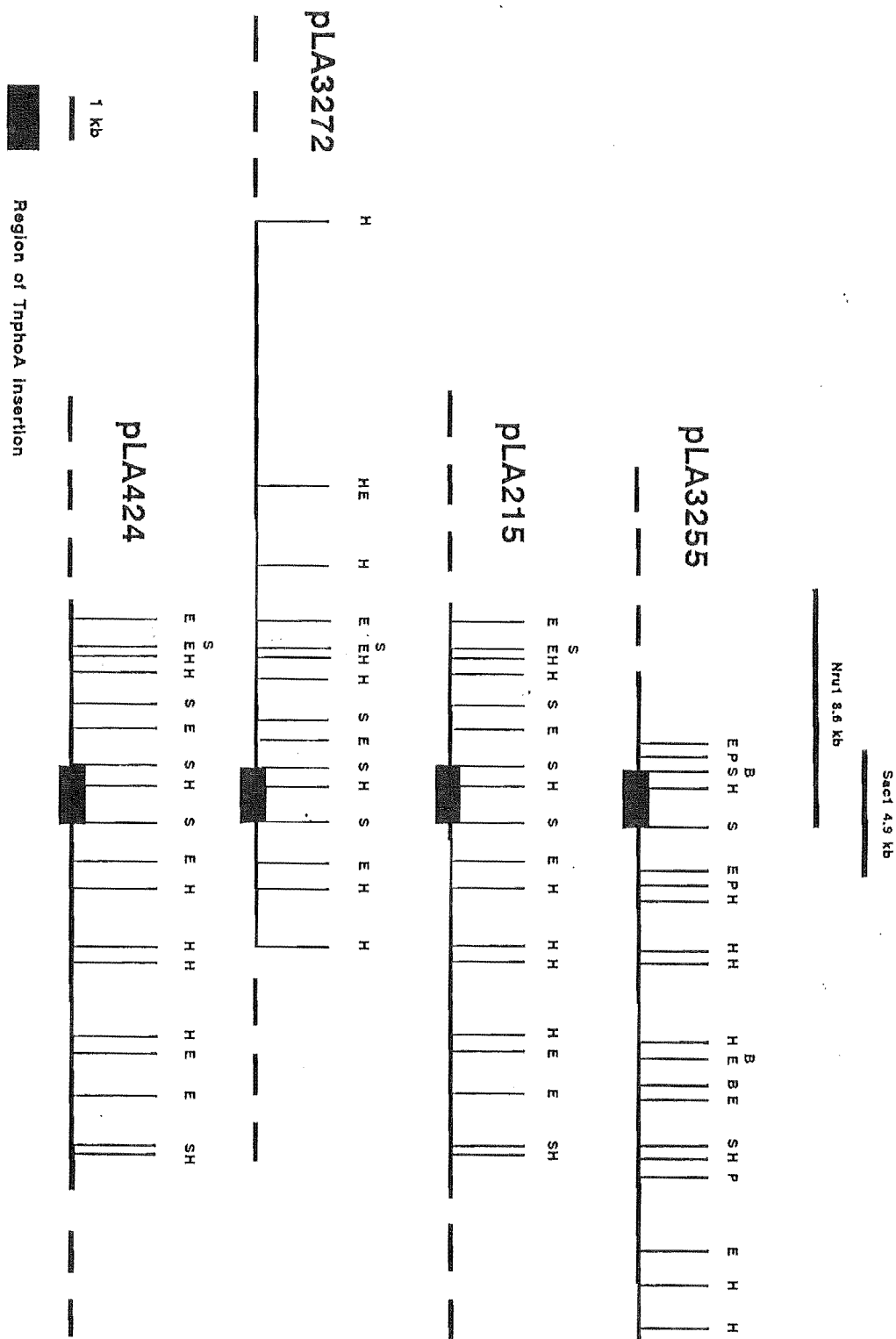


Figure 7.6: Restriction maps of complementing cosmids pLA215, pLA3255 and pLA3272 and partially complementing cosmid, pLA424, showing overlapping DNA regions. The region of Tn*phoA* insertions in the Ant⁻ mutants is indicated. Overlapping 8.6 kb *Nru*1 and 4.9 kb *Sac*1 restriction fragments, determined by Southern hybridisation, are also shown.

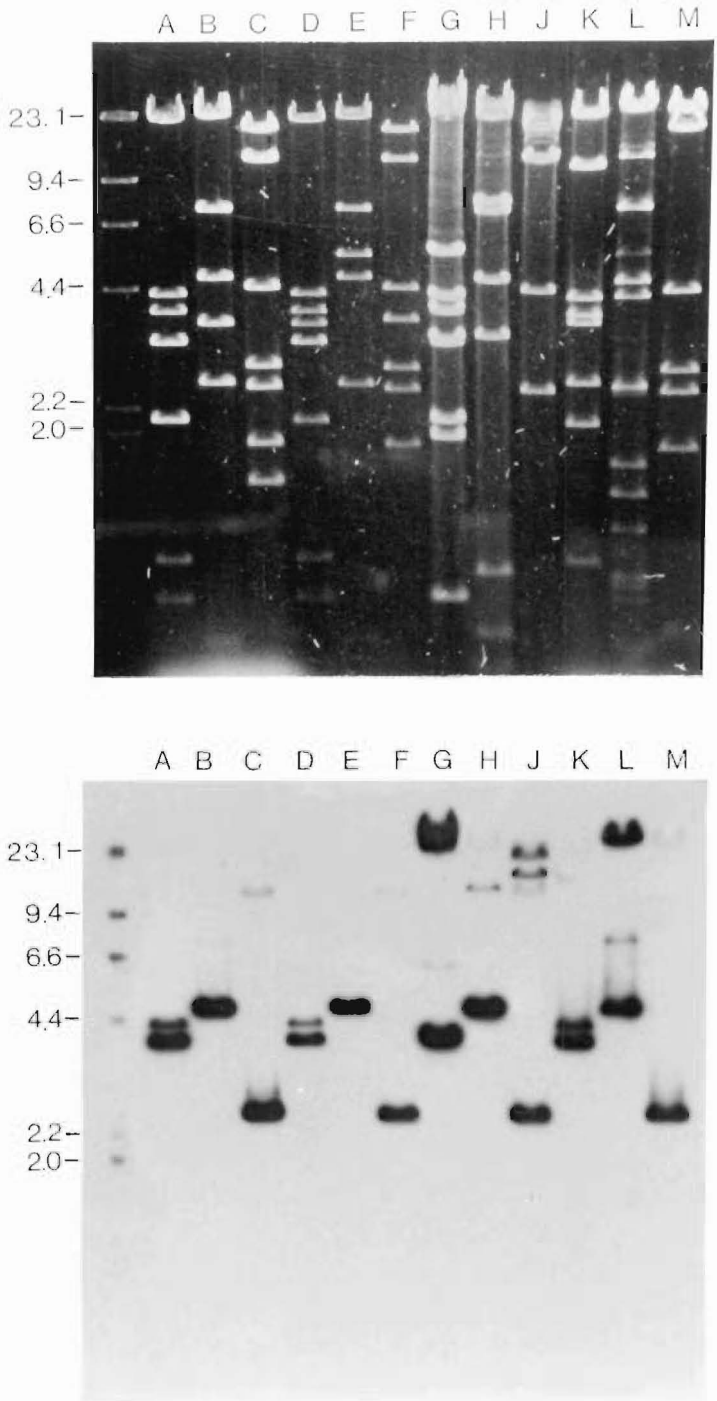


Figure 7.7: Gel electrophoresis of restriction digests of cosmid DNA and corresponding autoradiographs showing Southern hybridisation with a 2.4 kb *Eco*R1-*Sal*1 fragment probe from pLK2.

a). Lanes: pLA215 digested with A) *Hin*dIII, B) *Pst*1, C) *Sal*1; pLA424 digested with D) *Hin*dIII, E) *Pst*1, F) *Sal*1; pLA3255 digested with G) *Hin*dIII, H) *Pst*1, J) *Sal*1; pLA3272 digested with K) *Hin*dIII, L) *Pst*1, M) *Sal*1. Molecular weight markers are indicated on the left.

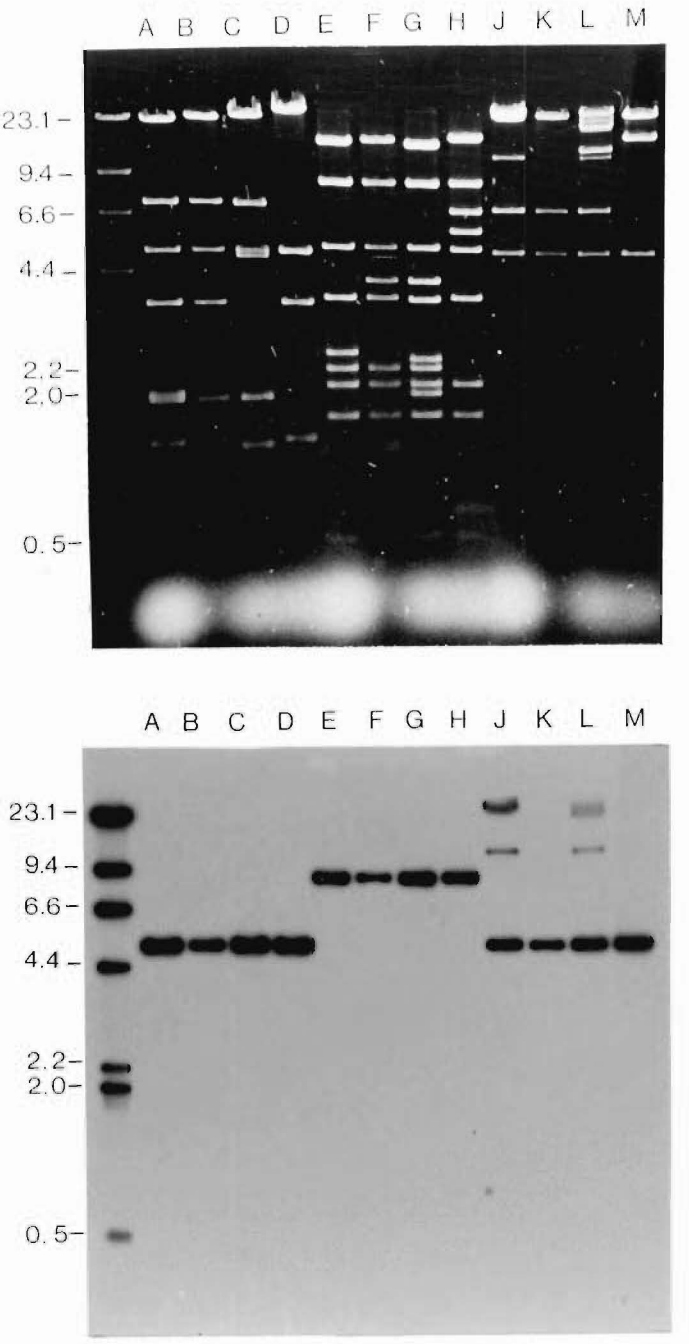
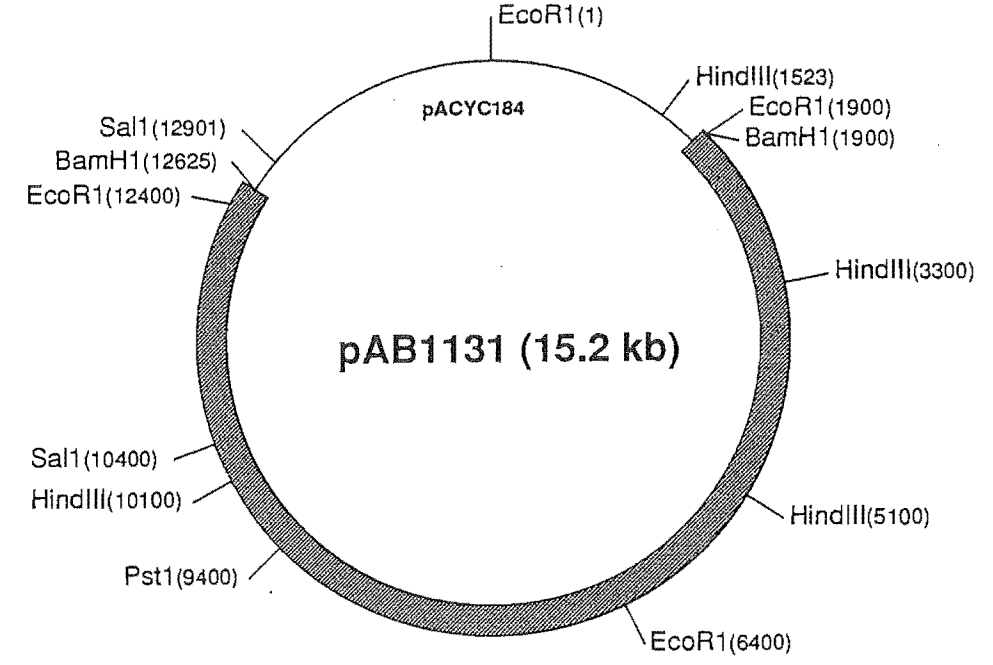


Figure 7.7 : (cont.)

b). Lanes: *Eco*R1-digested A) pLA215, B) pLA424, C) pLA3255, D) pLA3272; *Nru*1-digested E) pLA215, F) pLA424, G) pLA3255, H) pLA3272; *Sac*1-digested J) pLA215, K) pLA424, L) pLA3255, M) pLA3272. Lanes J and L contain partially-digested DNA. Molecular weight markers are indicated on the left.



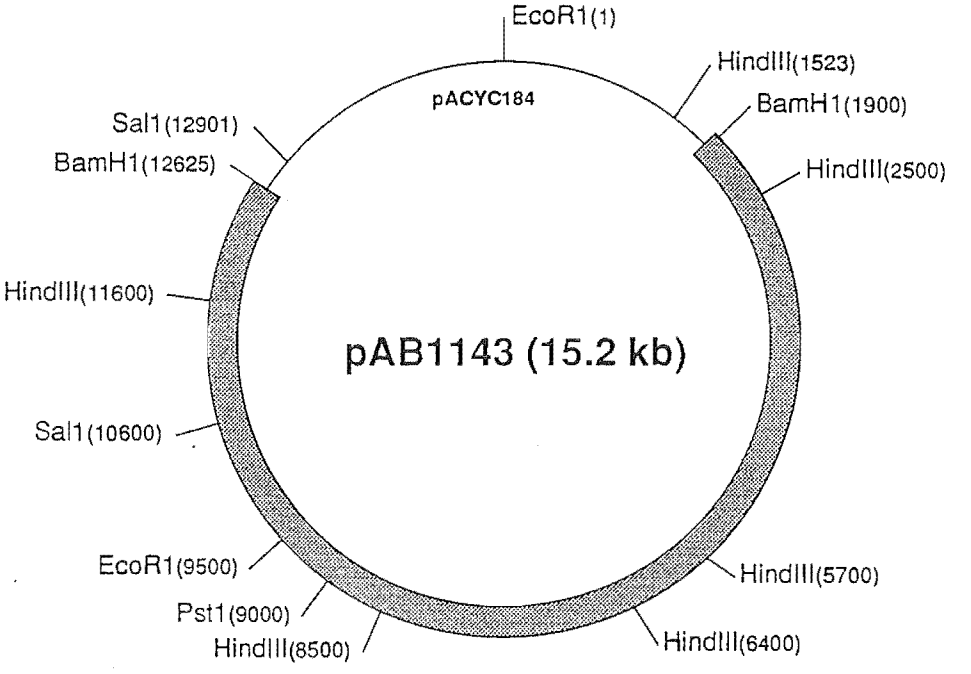
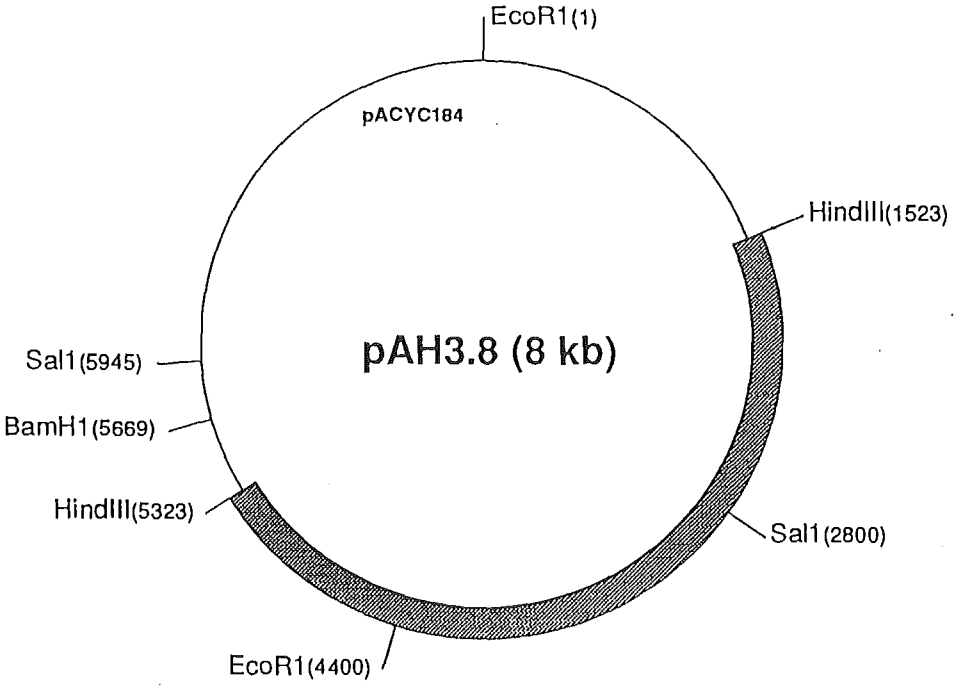


Figure 7.8: (Pages 111-114, incl.). Restriction maps of cosmid fragment subclones used for complementation assays. (Refer to Tables 7.2 and 7.10).



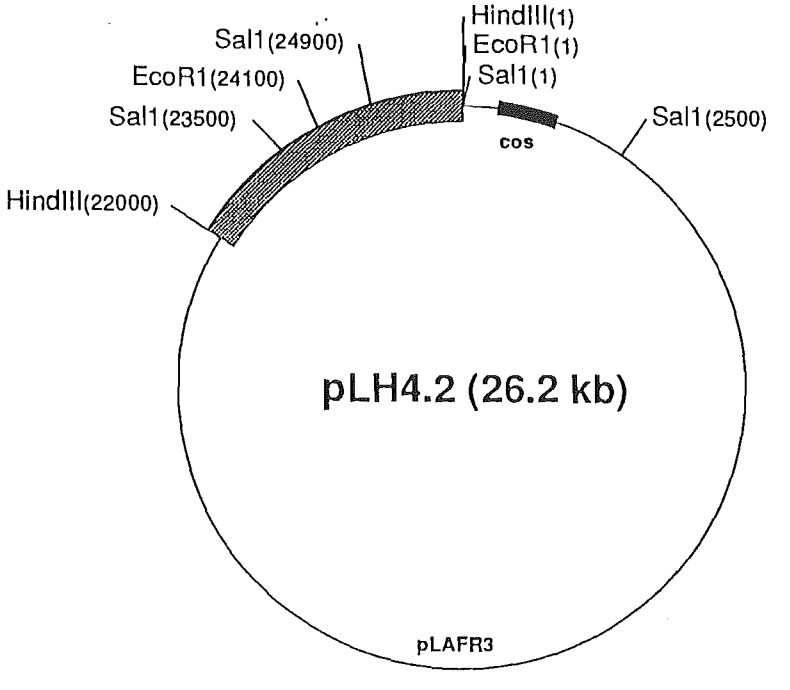
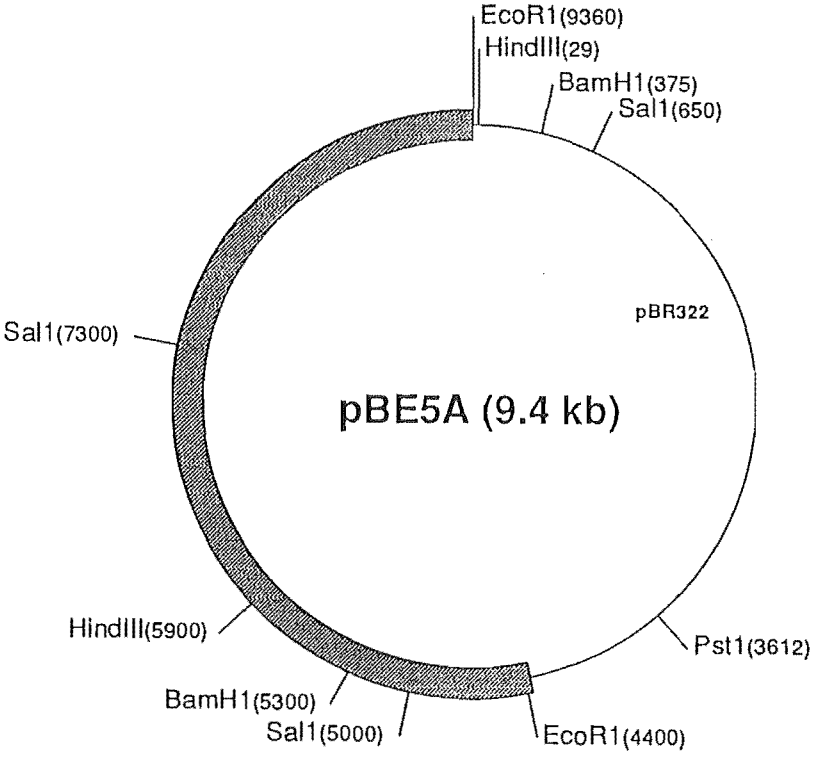


Figure 7.8: (cont.)



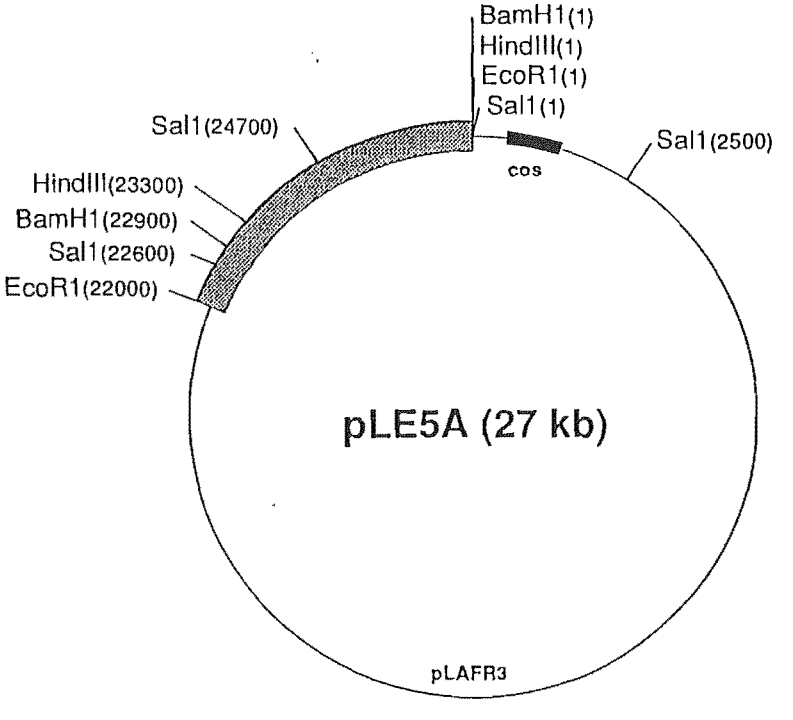


Figure 7.8: (cont.)

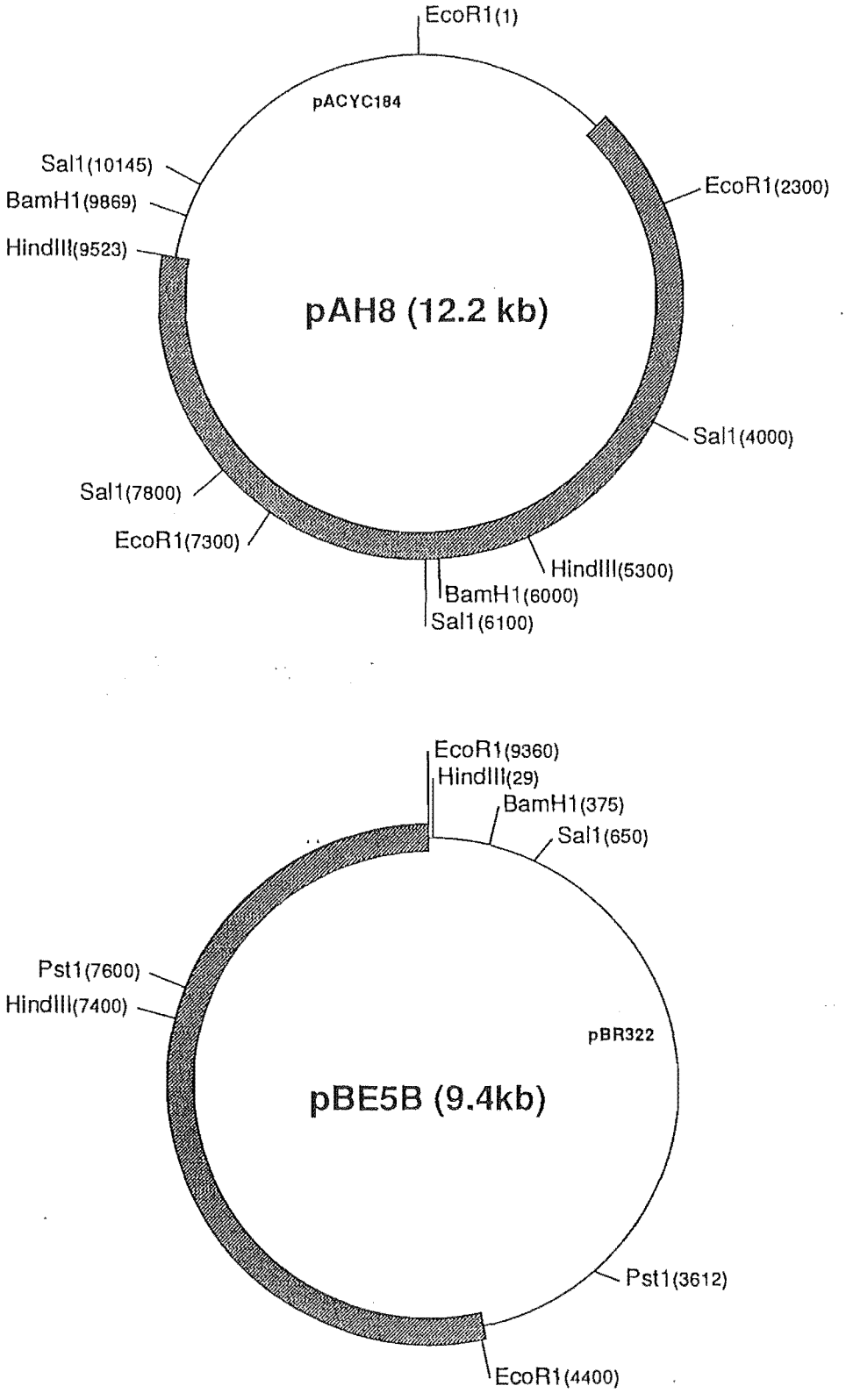
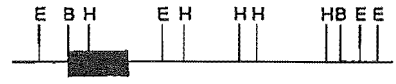
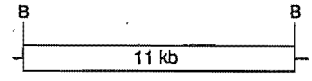
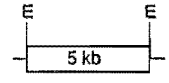
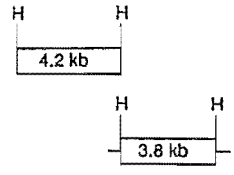
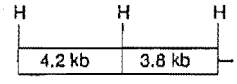
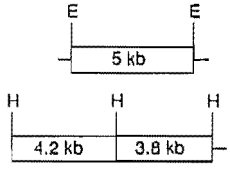
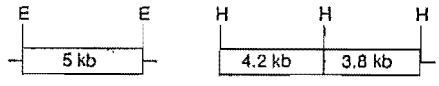


Figure 7.8: (cont.)

Table 7.10: Complementation assays with subcloned cosmid fragments.

Complementation assay	Orientation of fragment subclones	Fragment subclone[a]
		
1 (N.C.)[b]		pAB1143
2 (N.C.)		pLE5A or pBE5A
3 (N.C.)		pLH4.2 & pAH3.8 *in trans*
4 (W.C.)		pAH8
5 (W.C.)		pBE5A & pAH8 *in trans*
6 (C.)		pBE5B & pAH8 *in trans*
7 (N.C.)		pBE5B & pLE5A *in trans*

The region of overlap of complementing cosmids is shown at the top of the table, with the region of Tn*phoA* insertions highlighted. The orientation of each fragment subclone used for complementation assay is shown in relation to the overlapping region of the cosmids.

[a] See Table 7.2.
[b] (C.) indicates complementation, (W.C.) indicates weak complementation and (N.C.) indicates no complementation.

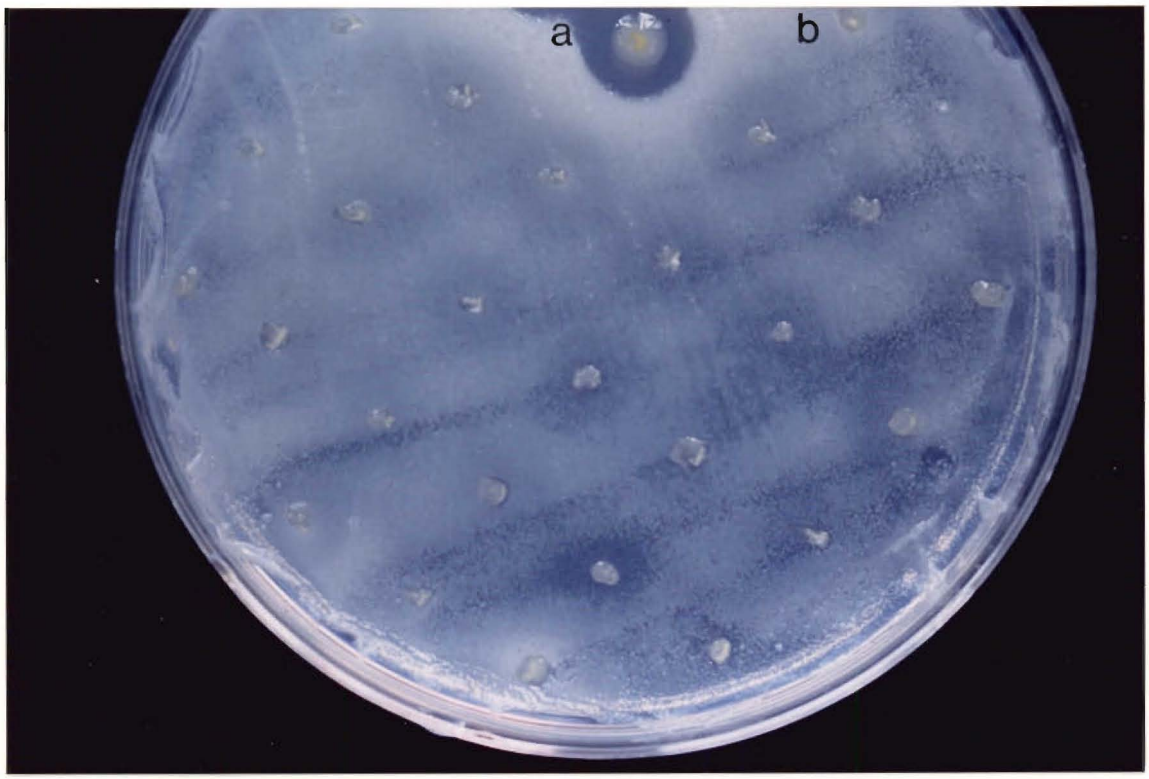


Figure 7.9: Weak complementation on HSN agar of Ant⁻ mutant EhA17g by cosmid fragment subclone pAH8. a) Eh1087, b) EhA17g, remainder of plate is EhA17g(pAH8).

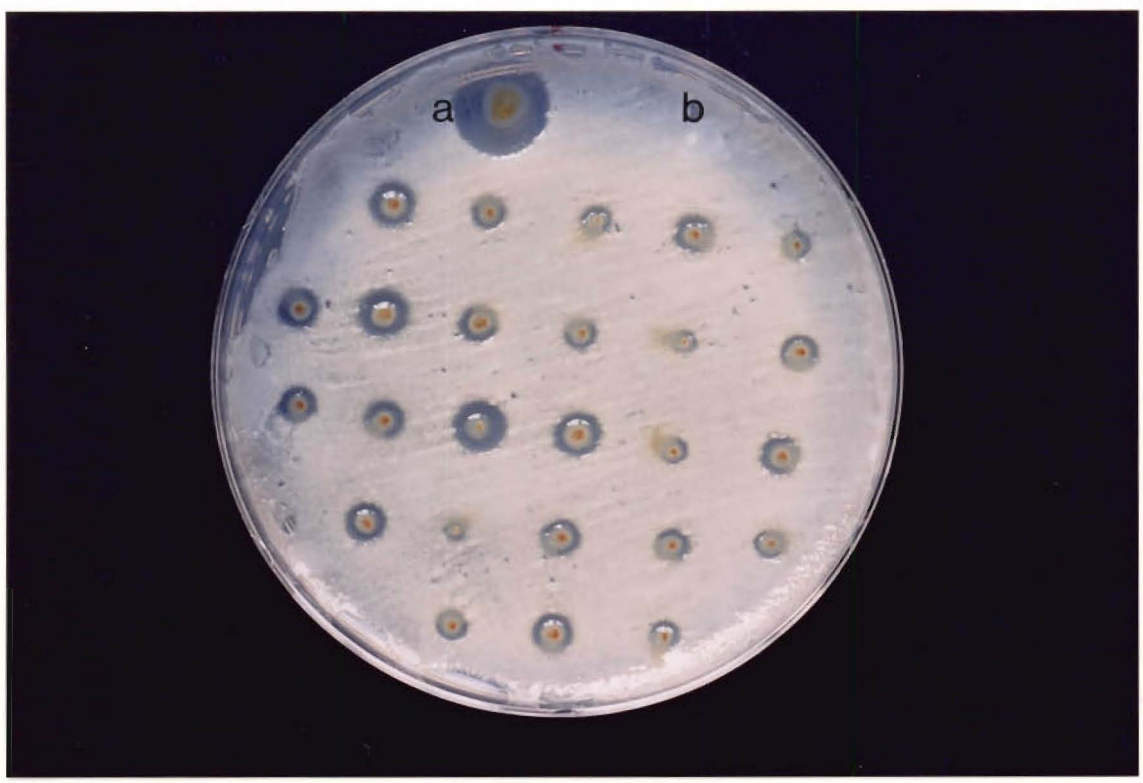


Figure 7.10: Complementation on HSN agar of Ant⁻ mutant EhA17g by cosmid fragment subclones, pAH8 and pBE5B, *in trans.* a) Eh1087, b) EhA17g, remainder of plate is EhA17g(pAH8, pBE5B).

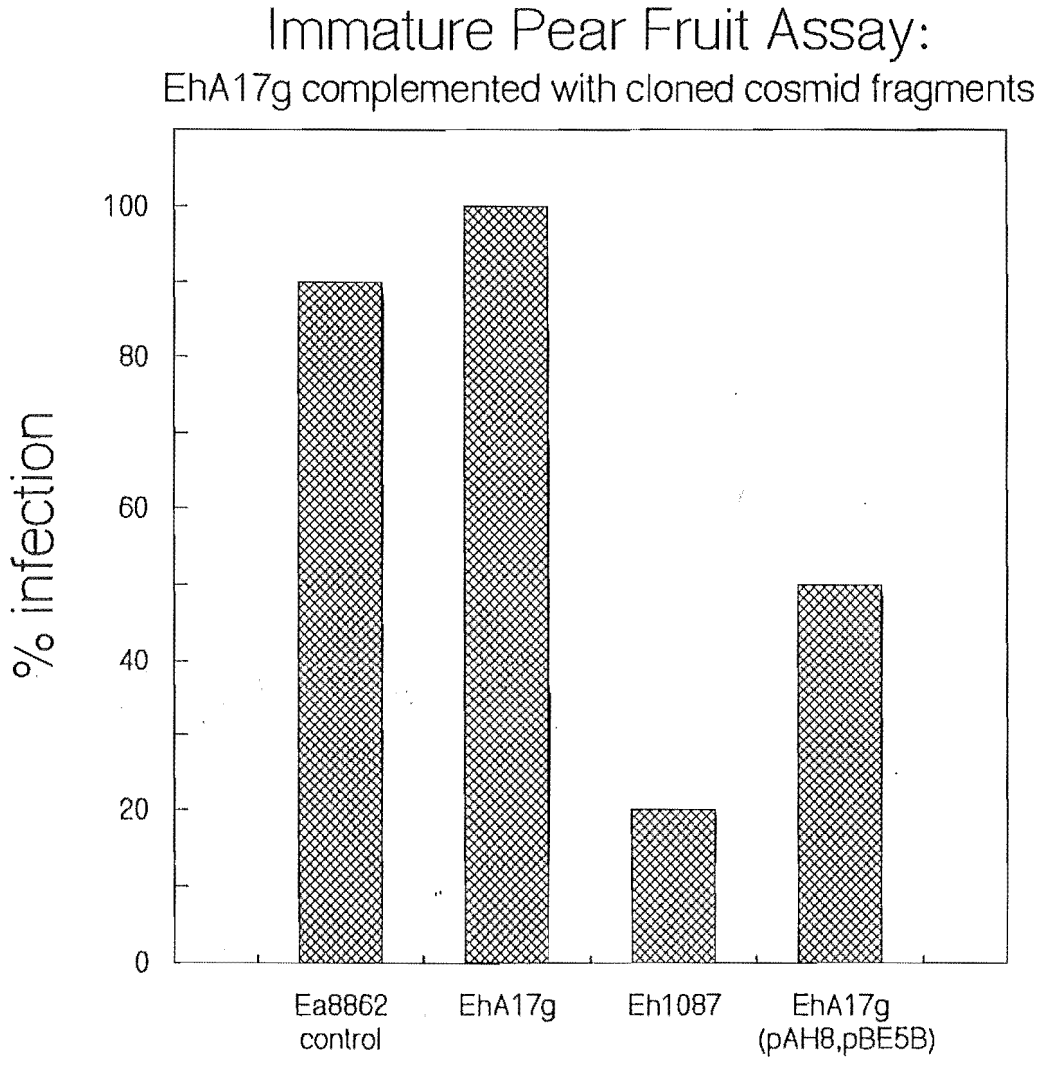


Figure 7.11: Complementation in immature pear fruit of Ant⁻ mutant EhA17g by cosmid fragment subclones, pAH8 and pBE5B, *in trans*. From left to right, % infection in pear fruit slices 3 days post inoculation is shown for Ea8862 alone; Ea8862 co-inoculated with EhA17g, Eh1087, EhA17g(pAH8,pBE5B), respectively. The results of a single bioassay are shown.

DISCUSSION

A genomic library of Eh1087 was constructed in cosmid pLAFR3. The efficiency of cosmid library preparation is much reduced by vector concatemerisation, resulting in cosmids lacking insert DNA. This problem is overcome by the preparation of individual vector arms (40, 73). To completely eliminate the formation of tandem vectors during ligation, vector arm preparation must be greater than 99.5% efficient (73). In the Eh1087 genomic library 22% of packaged cosmids lacked insert DNA, as BAP dephosphorylation was less than 99.5% efficient. When individual vector arms were not prepared, (ie. *Sau*3A insert DNA cloned directly into *Bam*H1-digested, dephosphorylated pLAFR3) 80% of colonies packaged contained tandem vectors without insert DNA (Results not shown).

The stability of pLAFR3 in recombinant *E. herbicola* was observed to decrease with time in laboratory culture and in frozen storage. At the time the Eh1087 genomic library was constructed and screened, pLAFR3 was observed to be stable in recombinant strains under the conditions tested and homologous recombination was a rare event (0-0.4%). With repeated laboratory subculture over many months, progressive loss of pLAFR3 from strains was indicated by the appearance of Tc^S, Ant^- strains, or only weakly Tc^R strains with a reduced inhibition of Ea8862. An increased frequency of homologous recombination in cosmid-carrying strains was also observed (69% and 78% in the strains tested) with the increasing instability of pLAFR3.

Plasmid pRK2, from which pLAFR3 is derived (41), has been reported to be unstable in *Xanthomonas vesicatoria* on agar medium in the absence of selection pressure, although stable up to six months *in planta* (90). It is possible that complementation may be enhanced by using a cloning vector other than pLAFR3. Recombination and loss of the complementing plasmid led to weak

complementation of pathogenicity mutants of *E. amylovora* by plasmid clones in pBR325 (10). Complementation was restored to wild-type levels when the complementing insert DNA was re-cloned into a plasmid, pCPP8, designed for stability in *E. amylovora* (10).

Alternatively, the creation of a RecA$^-$ strain of EhA17g, by identification of the "*recA*-like" genes of *E. herbicola* (79, 158) from the Eh1087 genomic library, mutational inactivation of these genes and recombination between the mutated cosmid and EhA17g, could facilitate complementation analysis .

Three cosmids, pLA215, pLA3255 and pLA3272, complemented Ant$^-$ mutants to the Ant$^+$ phenotype. The degree of restoration of inhibitory activity by complementing cosmids was varied, suggesting that the genetic backgrounds of the Ant$^-$ mutants differed from each other. Evidence from complementation analyses with cosmid fragment subclones indicated that the DNA region involved in antibiotic synthesis lies to the left of the region of Tn*phoA* insertions (Table 7.10). The non-overlapping region of the cloned DNA in the cosmids extends a distance of 3-14 kb beyond the LH end of the region of Tn*phoA* insertions (Figure 7.6). If the observed differences in the degree of complementation by the different cosmids is related to differences in the cloned DNA they carry, then this suggests that Tn*phoA* insertion in the mutants has caused polar mutations which may extend 3-14 kb to the left of the Tn*phoA* insertions.

"Partially complementing" cosmids were identified which restored a diminished inhibitory activity, in comparison to the wild-type. The restriction map of one of these "partially complementing" cosmids, pLA424, almost exactly coincided with the fully complementing cosmid, pLA215 (Figure 7.6). There may exist subtle differences between the cloned DNA of pLA215 and pLA424 at the LH junction between cosmid and insert DNA, that are not recognised by restriction analysis, but which influence complementation by the cosmids.

Numerous subcloned cosmid fragments were tested for complementation *in vitro*. An 11 kb *Bam*H1 fragment subclone, pAB1143, which incorporated most of the overlapping region of the complementing cosmids, failed to complement EhA17g (Table 7.10, Assay 1). An 8 kb *Hin*dIII fragment subclone, pAH8, extending in both directions beyond the region of Tn*phoA* insertions, resulted in a weak complementation (Table 7.10, Assay 4). Complementation *in trans* with the 5 kb *Eco*R1 fragment subclone, pBE5A, and pAH8, was assayed to determine whether or not weak complementation by pAH8 alone was due to minor base deletions or alterations at the junction point between its two constituent *Hin*dIII fragments, which could be compensated for *in trans* by pBE5A (Table 7.10, Assay 5). No enhancement of complementation was observed. Complementation by pAH8 was enhanced by the addition *in trans* of a second 5 kb *Eco*R1 subclone, pBE5B, that mapped approximately 6 kb to the left of the region of Tn*phoA* insertions (Table 7.10, Assay 6). This enhanced complementation could not be repeated with pBE5A and pBE5B subclones *in trans* (Table 7.10, Assay 7).

Weak complementation by pAH8 suggested either that the cloned 8 kb fragment was poorly expressed in pACYC184, or that the 8 kb fragment did not contain all the genetic information necessary to produce a fully active wild-type antibiotic molecule. Expression of cloned DNA under pACYC184 promoter control could be suppressed in some unknown way in the *E. herbicola* genetic background. Alternatively, the polar effects exerted by Tn*phoA* insertion in the mutant could extend beyond the region of the 8 kb *Hin*dIII fragment. For example, a final enzymatic step could be necessary to convert a partially active precursor antibiotic molecule to a molecule with full antibiotic activity. If such an enzyme was not encoded by the pAH8 subclone, pAH8-complemented mutants would produce only the antibiotic precursor molecule and would show reduced inhibitory activity for *E. amylovora*, in comparison to the wild-type.

Enhancement of pAH8 complementation by the fragment subclone, pBE5B, could be explained by proposing either that the pBE5B subclone codes for a positive regulator (possibly inactivated by upstream Tn*phoA* insertion in the mutant) which stimulates expression of the pAH8 clone, or alternatively, that the pBE5B subclone codes for a biosynthetic enzyme necessary to produce a fully active antibiotic molecule.

Antibiotic biosynthesis generally involves multistep pathways involving multiple gene clusters of up to 12 genes (3, 101). Mini-cell protein analysis of the gene products of pAH8 and pBE5B was carried out to further investigate the possible roles of these fragment subclones in complementation (Chapter 9).

CHAPTER 8

MINI-Tn*10* MUTAGENESIS OF pLA3255

SUMMARY

Mini-Tn*10* insertional mutagenesis of complementing cosmid pLA3255 was carried out in *E. coli* using a bacteriophage λ vector. When cosmids carrying mini-Tn*10* insertions were introduced into the mutant EhA17g background, an undefined recombination event occurred, giving rise to a non-complementing 4.3 kb plasmid, carrying Cm^R and Tc^R markers from the mini-Tn*10* construct and the cosmid, respectively. Because of this recombination, cosmids could not be screened for insertional inactivation of antibiotic biosynthetic gene(s).

INTRODUCTION

Cosmids from the Eh1087 genomic library were selected which restored antibiotic activity to Ant^- mutants of Eh1087. Complementation analyses were carried out with subcloned cosmid restriction fragments in order to more accurately define the coding region(s) involved in antibiotic biosynthesis. Two cosmid subclones, pAH8 and pBE5B, restored, *in trans*, a near wild-type antibiotic activity to Ant^- mutant EhA17g. The two subcloned fragments were non-contiguous and spanned a 17 kb region of DNA. The absence of appropriate restriction sites within this region meant that further definition of gene boundaries could not be carried out using restriction fragment subcloning.

Complementation by two non-contiguous subclones indicated that more than one coding region was involved in antibiotic biosynthesis. The large size of the DNA region involved (17 kb) suggested the possibility of a cluster of linked biosynthetic genes, not all of which had been identified. Therefore, mini-Tn*10* saturation mutagenesis of the entire complementing cosmids, pLA3255 and pLA3272, was attempted, to identify antibiotic encoding regions. Initial work with pLA3255 is reported here.

Mutagenesis of pLA3255 was carried out using a mini-Tn*10* construct (83). Mini-Tn*10* elements have a reduced insertion site specificity and an increased stability of insertion, due to the absence of the transposase gene, which is provided in *cis* on the delivery vehicle.

METHODS AND MATERIALS

Non-supressor *E. coli* strain W3110 (*sup*, F^-, λ^-; laboratory collection) was transformed with pLA3255 by electroporation. Preparation of cells for electroporation and electroporation conditions used were as outlined in Chapter 7. Electrotransformed W3110 were selected on LB agar, supplemented with Tc. Presence of the cosmid in Tc^R W3110 was confirmed by plasmid DNA mini-preparations using the method of Sambrook *et al.* (133).

Mini-Tn*10* derivative 105 (Cm^R) on λ delivery vehicle NK1324 was used, according to procedure 2 of Kleckner *et al.* (83), with the exception that selection plates were incubated at 37°C, not 39°C. Transpositions were carried out at an M.O.I. of 0.3. All colonies growing on the selective medium from 6 separate transposition experiments were pooled and plasmid DNA was prepared, according to the method of Sambrook *et al.* (133).

EhA17g was transformed with the pooled plasmid DNA by electroporation. EhA17g colonies were selected on LB agar supplemented with Rif, Km, Tc and Cm. Selected colonies of EhA17g(pLA3255::mTn*10*) were screened for inhibition of Ea8862 in an HSN soft agar overlay (For method see Chapter 3).

Mini-Tn*10* insertions in cosmids were mapped by comparing the restriction maps of mini-Tn*10*-mutated cosmids with those of non-mutated cosmids and identifying those fragments showing a 1.4 kb size increment, indicating mini-Tn*10* insertion.

RESULTS

An initial screening of 300 EhA17g(pLA3255::mTn*10*) colonies showed non-complementation in all colonies on HSN soft-agar overlays of Ea8862, indicating that all mini-Tn*10* insertions must lie in the DNA coding for antibiotic production. As mini-Tn*10* is not expected to show non-random insertions, the site of mini-Tn*10* insertion was checked for 5 of the transformed colonies. *Sal*1 digested cosmid DNA from these colonies is shown in Figure 8.1. In all five samples, the expected pLA3255 *Sal*1 digest pattern, modified slightly by the 1.4 kb mini-Tn*10* insert, was absent and a single band at 4.3 kb was observed.

This 4.3 kb DNA was used to transform DH5α (Figure 8.2), to give Cm^RTc^R colonies, indicating the presence of a circular replicating plasmid carrying Cm^R and Tc^R genes. These findings suggested a recombination event was occurring in the EhA17g background.

In order to confirm that the observed 4.3 kb plasmid arose from recombination in EhA17g and did not occur during mini-Tn*10* mutagenesis in *E. coli*, EhA17g was transformed with six separate pLA3255::mTn*10* molecules with known mapped

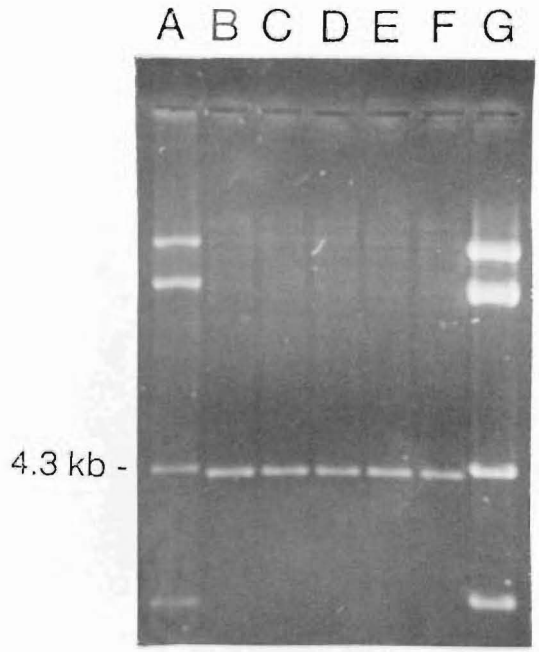


Figure 8.1: DNA from EhA17g transformed with pLA3255::mTn*10*. Lanes: *Sal*1-digested DNA of A) pLA3255, B) - F) 5 separate strains of EhA17g transformed with pLA3255::mTn*10*, G) pLA3255.

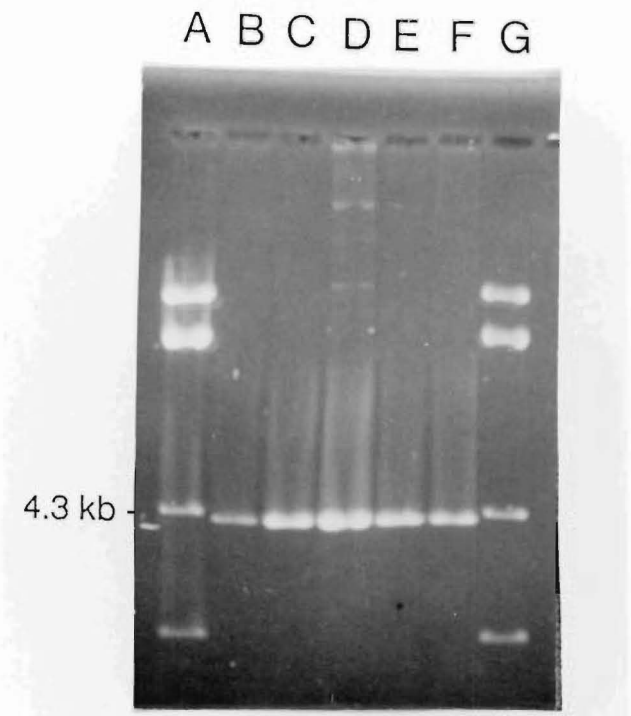


Figure 8.2: DNA from *E. coli* DH5α transformed with 4.3 kb DNA from EhA17g. Lanes: Sal1-digested DNA of A) pLA3255, B) - F) 5 separate strains of DH5α transformed with DNA of Figure 8.1 lanes B) - F), respectively, G) pLA3255.

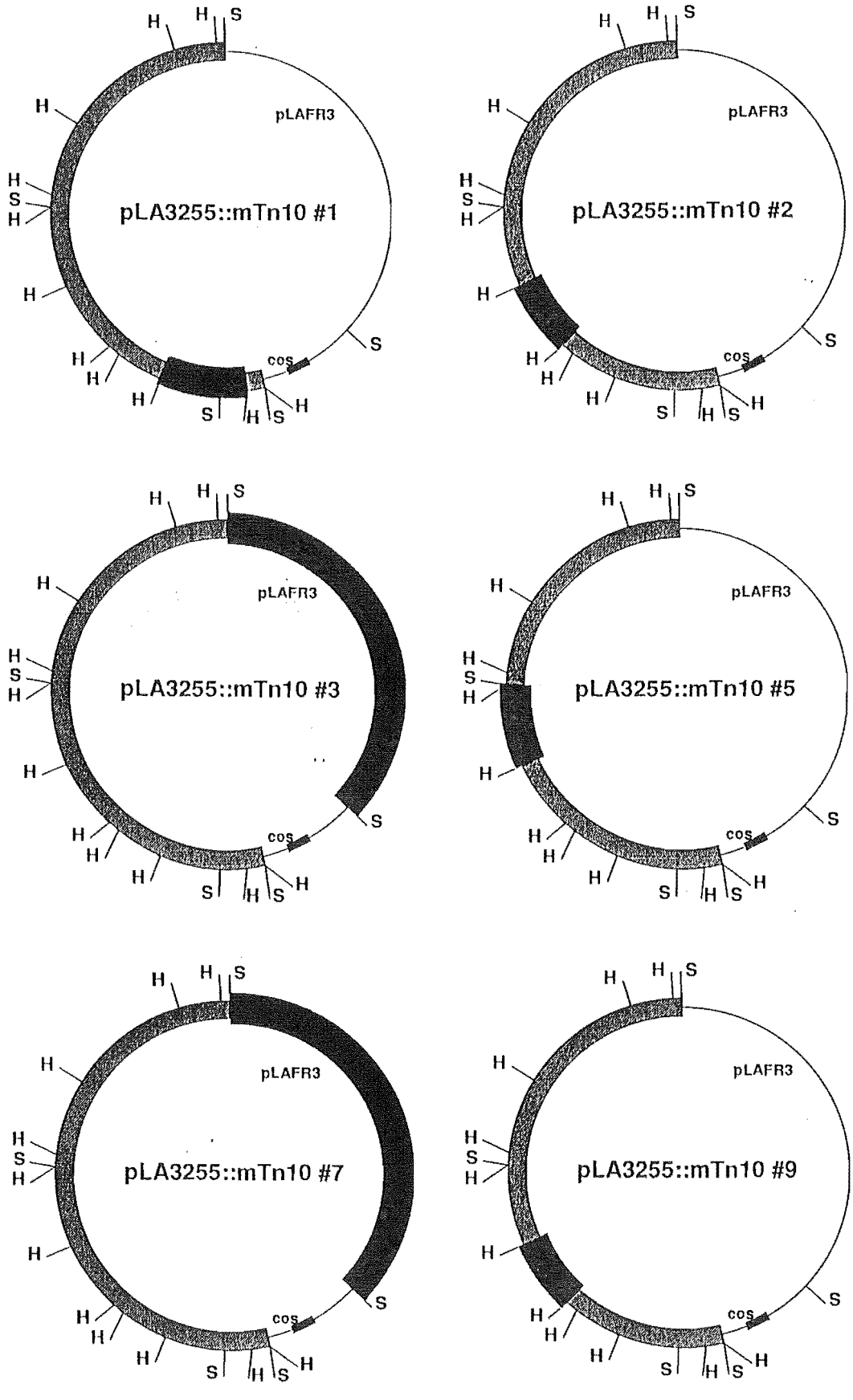


Figure 8.3: Restriction maps of pLA3255::mTn*10* molecules showing different sites of mini-Tn*10* insertion. Restriction fragments containing inserted mini-Tn*10* are shown in bold.

sites of mini-Tn*10* insertion (Figure 8.3). In two of the six mapped cosmids mini-Tn*10* insertions were located in the pLAFR3 vector (#3 & #7) and in the remaining four cosmids mini-Tn*10* was located in the cloned insert DNA (#1, #2, #5 and #9). The plasmid species present in EhA17g transformed with these mutated cosmids are shown in Figure 8.4. Only one of the transformed strains (#7) showed the presence of the transforming pLA3255::mTn*10* molecule, while the others again showed a 4.3 kb DNA band.

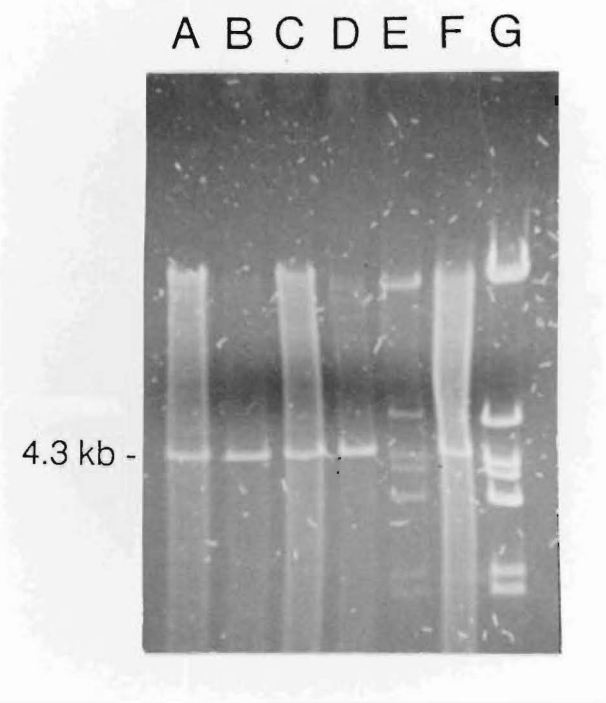


Figure 8.4: DNA from EhA17g transformed with pLA3255::miniTn*10*. Lanes: A)-F) *Hin*dIII-digested DNA from EhA17g transformed with pLA3255::mTn*10* #1, #2, #3, #5, #7 and #9, respectively, from Figure 8.3, G) *Hin*dIII-digested pLA3255.

Colonies of EhA17g (pLA3255::mTn*10*) #7 remained Ant$^+$, as expected, while strains carrying the 4.3 kb plasmid failed to inhibit Ea8862 on HSN agar.

DISCUSSION

EhA17g strains carrying mini-Tn*10* mutated pLA3255 underwent an undefined recombination event which gave rise to a replicating 4.3 kb plasmid carrying Tc^R and Cm^R markers. This recombination did not involve restoration of the Ant^+ phenotype to the mutant by exchange of homologous DNA between the cosmid and the EhA17g genome. Recombination occurred under full antibiotic selection pressure (Rif, Tc, Km and Cm). Although recombination between pLA3255 and the EhA17g genome occurred at high frequncy in the absence of selection pressure (Chapter 7), the cosmid was otherwise stably maintained in mutant strains with full selection pressure, suggesting that the presence of the mini-Tn*10* transposon in the cosmid may have stimulated recombination in some unknown way.

The origin of replication of the recombinant 4.3 kb plasmid was probably sourced from pLAFR3 as essential replication regions flank the Tc^R gene in the vector (41). Alternatively, the replication gene(s) could have come from the indigenous plasmid of Eh1087. If the 4.3 kb plasmid contained the origin of replication from the Eh1087 indigenous plasmid, it could provide a vehicle for construction of a new plasmid cloning vector, based upon the Eh1087 plasmid, which may be more suited to use in *E. herbicola* than other commonly used plasmids. The source of the replication gene(s) in the 4.3 kb plasmid was not investigated in this study.

Bacteriophage Mu has been used to generate pathogenicity mutants of *E. amylovora* (160) and *E. chrysanthemi* (5, 46) and it is possible that cosmid saturation mutagenesis with a mini-Mu construct (7, 25), rather than mini-Tn*10*, may avoid the problems of recombination encountered in this study. Use of a mini-Mud-*lac* element (25) to generate *lac* gene fusions could facilitate identification of DNA coding regions, by providing visual selection for in-frame mini-Mu insertions and by indicating the direction of transcription of gene fusions.

Because of the inability to analyse mini-Tn*10* mutated cosmids for complementation in EhA17g, this experimental approach was terminated and the protein products expressed by the complementing cosmid fragment subclones, pAH8 and pBE5B, were investigated in an *E. coli* mini-cell system (Chapter 9).

CHAPTER 9

PROTEIN ANALYSIS OF COSMID FRAGMENT SUBCLONES IN THE E. coli MINI-CELL SYSTEM

SUMMARY

E. coli mini-cell protein analysis of the gene products encoded by recombinant plasmids pBE5B and pAH8 showed that pBE5B expressed three proteins of 20, 34 and 41 kd and pAH8 coded for two proteins, 28 and 39 kd in size. The possible functions of these proteins in antibiotic biosynthesis in Eh1087 is discussed.

INTRODUCTION

Because of the inability to accurately define the locus of interest in the complementing cosmids using mini Tn*10* mutagenesis, it was decided to analyse the gene products of complementing cosmid fragment subclones, pBE5B and pAH8, using *E. coli* mini-cell analysis.

Mini-cells contain little or no chromosomal DNA due to aberrant cell division during the growth cycle of mini-cell producing mutant strains of *E. coli*. (1, 31, 53). Transcription and translation are still functional, as mini-cells retain cellular protein and RNA. Recombinant plasmids can be introduced into mini-cell producing strains via conjugation, transformation or infection with bacteriophage. Use of the mini-cell system facilitates the analysis of gene products expressed by recombinant plasmids as expression of chromosomally encoded proteins is absent.

In this study, proteins produced in mini-cells containing pBE5B and pAH8, singly and together, were radio-labelled by incorporation of ^{35}S-labelled amino acid precursors, and analysed by SDS-PAGE.

METHODS AND MATERIALS

Preparation of *E. coli* mini-cells

E. coli mini-cell producing strain P678-54 (1) was used. The genotype for this strain is F$^-$ thr$^-$ leu$^-$ *supE lacY tonA* galB- mal$^-$ xyl$^-$ ara$^-$ mtl$^-$ min$^-$. *E. coli* P678-54 cells were prepared for electroporation and electroporated with plasmids pBR322, pACYC184, pBE5B and pAH8, using methods described in Chapter 7. The presence of plasmids in electroporated strains was confirmed by plasmid DNA mini-preparations by the method of Sambrook *et al.* (133).

Plasmid-containing mini-cells were purified by differential centrifugation and differential rate sedimentation in sucrose gradients (53). LB broth cultures (300 ml), supplemented with the appropriate antibiotics, of each mini-cell strain were grown overnight at 37°C to an $O.D._{600}$ = 0.8 - 1.2. Vegetative cells were sedimented by centrifugation in a Sorvall GSA rotor at 2000 rpm for 10 min at 4°C. The surface of the vegetative cell pellet was gently washed with a little retained supernatant to remove any co-sedimented mini-cells and the pellet wash was pooled with the supernatant fraction. The supernatant was re-centrifuged at 10,000 rpm for 10 min to sediment mini-cells. The mini-cell pellet was thoroughly resuspended in 2 ml BSG buffer (NaCl 8.5g.l^{-1}, KH_2PO_4 0.3 g.l^{-1}, Na_2HPO_4 0.6 g.l^{-1}, gelatin 0.1 g.l^{-1}) at room temperature and layered onto a 35 ml continuous linear sucrose gradient in a clear 50 ml plastic centrifuge tube. Sucrose gradients were prepared by freeze-thawing 35 ml sterile 20% glucose (w/v) in M63 medium (108) in the centrifuge tube. Sucrose gradients were centrifuged at 5,000g for 15 min at 10°C in a benchtop centrifuge (Sigma), using a swing out rotor. The

mini-cell (upper) band was harvested (10 ml) with a sterile pasteur pipette, leaving the lower portion of the band in the tube, and slowly diluted with an equal volume of BSG buffer, before re-sedimenting the cells by centrifugation as before. Mini-cells were then re-cycled over another sucrose gradient and re-sedimented as above. The mini-cells harvested from the second sucrose gradient were gently resuspended in BSG buffer (10 ml) at room temperature and the $O.D._{600}$ measured. Mini-cells were re-sedimented and resuspended in sterile M63/30% glycerol (v/v) to give an $O.D._{600} = 2$. Aliquots (100 μl) were stored frozen at -80°C. To check the quality of each mini-cell preparation, an aliquot (10 μl) of cells was plated onto LB agar, supplemented with the appropriate antibiotics, and incubated overnight at 37°C to determine the level of vegetative cell contamination of mini-cells. Fewer than 1,000 colonies per 10 μl was considered an acceptable level of contamination.

Labelling of proteins synthesised in mini-cells

Frozen mini-cells (100 μl aliquots) were thawed on ice for 10-20 min and 900 μl M63 labelling buffer (M63 medium supplemented with amino acids, with the exception of methionine) was added to each 100 μl. Mini-cells were pre-incubated in a small culture flask (25 ml) for 20-40 min at 37°C with orbital shaking and ^{35}S-methionine (20 μCi.ml^{-1}) was added to each flask. Flasks were incubated a further 30-45 min at 37°C. Labelled cells were transferred to an eppendorf tube and collected by centrifugation 2 min at 12,000g. The cell pellet was resuspended in 60 μl storage buffer (Na_2HPO_4 7 g.l^{-1}, KH_2PO_4 3 g.l^{-1}, NaCl 4 g.l^{-1}, $MgSO_4$ 0.1 g.l^{-1}) and stored frozen at -20°C.

SDS-PAGE of mini-cell proteins

Labelled mini-cells were thawed, diluted with an equal volume of 2x Laemmli sample buffer (89) and boiled for 3 min prior to loading 60 μl volumes into each well of a polyacrylamide gel. Proteins were analysed by SDS-PAGE using the Laemmli system (88). Gradient gels (10-20% polyacrylamide, 0.5 mm thickness)

were electrophoresed overnight at 50 V. Low molecular weight protein markers (BioRad) were used. Gels were stained in Coomassie blue, then de-stained and soaked in Amplify (Amersham) solution for 30 mins and vacuum-dried onto filter paper using a Model 443 slab drier (BioRad) for 5 hours at 60°C. Dried gels were autoradiographed as previously described (Chapter 6).

RESULTS

SDS-PAGE of proteins produced by *E. coli* mini-cells containing cosmid fragment subclones pBE5B and pAH8 is shown in Figure 9.1. pBE5B coded for three proteins of 20, 34 and 41 kd and pAH8 coded for two proteins, 28 and 39 kd in size. When both plasmids were present in the same mini-cells all five protein bands were produced, although expression of the pBE5B-encoded proteins was much reduced. No unique protein bands were seen in mini-cells containing both plasmids.

DISCUSSION

Mini-cell protein analysis of the recombinant plasmid pAH8 showed that this 8kb *Hin*dIII fragment codes for two proteins, 39 kd and 28 kd in size. This fragment clone weakly restored antibiotic activity to Ant$^-$ mutant EhA17g (Chapter 8), so at least one of the two encoded proteins must be essential for Eh1087 antibiotic activity.

Eh1087 produces a β-lactam antibiotic (Chapter 5). Microbially produced β-lactam antibiotics fall into five classes, according to the molecular structure associated with the β-lactam ring: penam (penicillins) and cephem (cephalosporins and cephamycins) antibiotics and the more recently discovered bacterial clavam, carbapenum and monobactam antibiotics (3). β-lactamase (penicillinase/cephalosporinase) digestion inactivated the antibiotic of Eh1087.

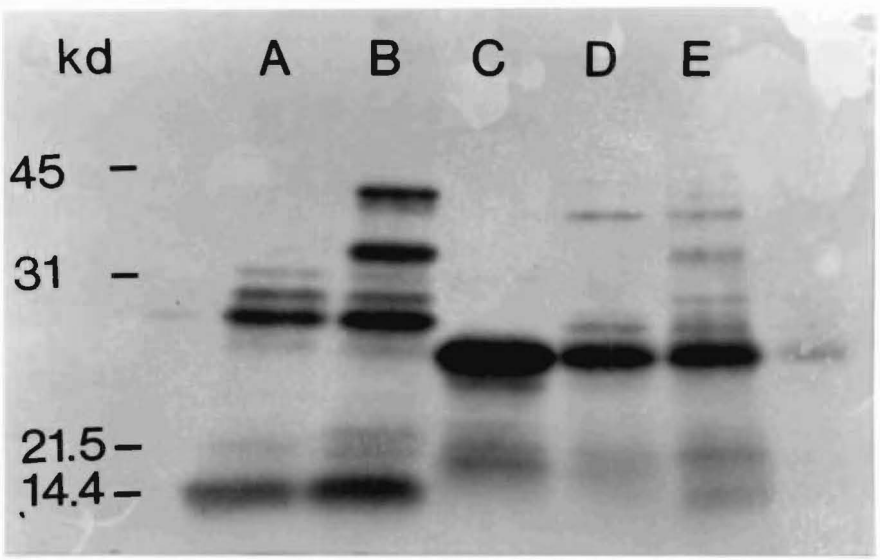


Figure 9.1: SDS-PAGE of proteins in *E. coli* mini-cells carrying A) pBR322, B) pBE5B, C) pACYC184, D) pAH8 and E) pAH8 and pBE5B. Molecular weight markers (kdal) are shown on the left.

Cephamycins and many of the novel bacterial β-lactam antibiotics (clavams, carbapenums and monobactams) have been shown to be only poorly hydrolysed by a variety of β-lactamases (3, 113). As bacteria do not produce penicillins as their end-product (3), this indicates the possibility of a cephalosporin-like activity being produced by Eh1087.

The biosynthetic pathways of penam and cephem antibiotics are well characterised at an enzymatic level and most of the biosynthetic genes have been isolated and cloned (3). Penicillins and cephalosporins are produced by essentially the same biosynthetic pathways in eukaryotes and prokaryotes and the genes involved are closely related. The common precursor molecule to the synthesis of these antibiotics is a linear tripeptide d(L-a-aminoadipyl)-L-cysteinyl-L-valine (ACV). ACV is cyclised by the enzyme isopenicillin N synthase (IPNS) to form isopenicillin N. In cephalosporin- and cephamycin-producing organisms isopenicillin N is isomerised to penicillin N by epimerase, then the thiazolidine ring of penicillin N is oxidised to a dihydrothiazine ring by expandase to give deacetoxycephalosporin C (DAOC). Hydroxylase enzyme then converts DAOC to deacetylcephalosporin C (DAC). From this intermediate, the biosynthetic pathways of cephalosporins and cephamycins branch. Cephalosporin C synthetase catalyses the conversion of DAC to cephalosporin C. The conversion of DAC to cephamycins requires a further three enzymatic steps.

The 39 kd protein produced by the pAH8 clone has the same molecular weight as IPNS (3, 101). The gene encoding IPNS, *pcbC*, has now been isolated and cloned from numerous organisms, including fungi and Gram positive and Gram negative bacteria (3). Sequence comparisons of these genes show 60-80% homology, indicating a highly conserved enzyme activity. Furthermore, DNA hybridisation analyses indicate presence of the IPNS-encoding gene in a wide range of Gram positive and Gram negative bacteria. Hybridisation of the pAH8 clone with a heterologous DNA probe of the *pcbC* gene could identify whether or not a related

pcbC gene from *E. herbicola* has been cloned.

The *Eco*R1 5 kb fragment subclone coded for three proteins, 20, 34 and 41 kd, respectively, and enhanced the complementation of Ant⁻ mutant, EhA17g, by pAH8. This DNA fragment was not contained in all the complementing cosmids and mapped approximately 6 kb away from the Tn*phoA*-mutated region. This suggests that the enhancement of complementation by this fragment may be due to a gene dosage effect, leading to increased levels of expression of gene product(s) involved in Eh1087 antibiotic expression that are actually still produced in the Ant⁻ mutant.

This clone may code for a regulatory protein, perhaps similar to the positive effector molecules of antibiotic-producing *Streptomyces* species (3, 101). Very little is known about the regulation of gene expression in antibiotic biosynthesis. Transcription of pBE5B genes is greatly reduced by the presence of pAH8 in the same cell, tempting speculation that a feedback regulation by the product(s) of the pAH8 clone could be occurring.

Alternatively, the *Eco*R1 5 kb fragment subclone may code for other enzymes involved in Eh1087 antibiotic synthesis. The genes coding for epimerase, expandase and hydroxylase enzymes, *cefD*, *cefE* and *cefF*, respectively, have been cloned from *Streptomyces clavuligerus*. These genes are linked on a 6 kb region of DNA within the antibiotic biosynthetic gene cluster of this organism and map approximately 5 kb upstream from the *pcbAB* gene, which encodes ACV synthetase and which lies immediately upstream of the *pcbC* gene. The molecular weights of *S. clavuligerus* purified epimerase (47 kd), expandase (29.5 kd) and hydroxylase (26.2 kd) enzymes approximately match those of the pBE5B-encoded proteins (41, 34 and 20 kd). (Recombinant *E. coli*, expressing *S. clavuligerus cefD*, *cefE* and *cefF* genes, produce enzymatically active proteins of 43.5, 34.6 and 34.6 kd, respectively). The similarites of the proteins produced and the arrangement of

the corresponding genes, suggest that DNA hybridisation of pBE5B DNA with heterologous probes for *cefD*, *cefE* and *cefF* genes may determine whether or not this fragment subclone contains related biosynthetic genes.

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APPENDIX A

Secondary metabolite medium

Yeast extract	0.5	g
Glucose	25	g
KH_2PO_4	13.6	g
citric acid	0.4	g
Na_2SO_4	0.05	g
Trace elements	0.05 ml of stock soln.	
Agar	15	g
Distilled water	1000	ml

Adjust pH to 6.5 with KOH and autoclave.

Trace elements 1,000x stock solution

$MnSO_4.H_2O$	100	mg
H_3BO_3	30	mg
$ZnSO_4.7H_2O$	30	mg
$Na_2MoO_4.2H_2O$	2.5	mg
$CuSO_4.5H_2O$	2.5	mg
$CoCl_2.6H_2O$	2.5	mg
Distilled water	100	ml

Store trace element solution at 0 - 4°C.

APPENDIX B

WEATHER DATA

Loburn, CANTERBURY, 1989 (Orchards A and B)

DATE OCT	TEMPERATURE MIN	MAX	AVG	RAINFALL (mm)	REL. HUMIDITY % RH at noon
1	13	22	17.5	trace	58
2	10	22	16	nil	56
3	9	19	14	trace	82
4	6	13	9.5	10	77
5	6	13	9.5	0.5	67
6	4	9		6	67
7	7	10	8.5	10	
8	8	12	10	43	96
9	8	17	12.5	trace	79
10	6	21	13.5	nil	43
11	8	17	12.5	2	66
12	4	16	10	trace	67
13		17		nil	63
14	8	22	15	nil	
15	10	18	14	3	60
16	10	18	14	3	73
17	7	14	10	24	73
18	5	12	8.5	4	71
19	5	13	9	trace	69
20		16	8	nil	61
21	11	18	14.5	8.2	
22	10	14.5	12.4	20	88
23	10	18	14	trace	
24	10	21	16	nil	
25	8	19	13	nil	
26	8	19	13	nil	
27	10	23	16	nil	
28	10	26	18	nil	
29	14	22	18	trace	66
30	9	18	13.5	1	78
31	12	24	18	nil	37
NOV					
1	10	25	17.5	trace	47
2	7	16	11.5	trace	69
3		17		nil	57
4	9	18	13.5	nil	
5	7	19	13	nil	69
6	13	27	20	nil	38

Rainfall data courtesy Christchurch Meteorological Office, Rangiora station. Weather data, 22 - 27 October, courtesy Lincoln University, Lincoln research orchard. All other weather data courtesy Christchurch Meteorological Office, Christchurch airport.

Havelock North, HAWKES BAY 1990 (Orchards C and D)

DATE OCT	TEMPERATURE MIN	MAX	RAINFALL (mm)
1	7.3	10.6	12.2
2	5.5	15.2	nil
3	1	18.8	nil
4	1.5	19	nil
5	9	20	8.4
6	2.6	20.7	nil
7	13.1	15.8	nil
8	1.5	nil	
9	18.9	nil	
10	4.3	19.4	nil
11	8.2	23	nil
12	11.1	25.5	2.6
13	7	25.5	nil
14	5.9	22	1
15	14.2	20.6	1.6
16	12.6	14.8	4.2
17	9.3	13.6	0.2
18	7.3	17.2	0.2
19	7	21.9	2.6
20	9.1	21.2	nil
21	8.3	23	nil
22	14	24.2	nil
23	7.3	16.6	8.8
24	12.1	17.4	4
25	11.8	19.9	nil
26	12.9	22.6	nil
27	8.4	24.3	nil
28	9.9	13.1	4
29	2	18.1	nil
30	7	21.1	nil
31	11.1	20.6	1.2
NOV			
1	2.6	17.2	nil
2	3.7	17.1	nil
3	3.1	21.9	nil
4	9.2	21	nil
5	8.3	24.7	nil
6	4.2	15.4	nil
7	2.5	20.7	nil

Weather data courtesy New Zealand Institute for Horticulture and Food Research, Havelock North station.

Lincoln, CANTERBURY 1991 (Eh1087 orchard survival trials)

DATE OCT	TEMPERATURE MIN	MAX	AVG	RAINFALL (mm)	REL. HUMIDITY % RH at noon
1	5.8	11.1	8.1	0.0	41
2	1.8	13.0	8.1	2.4	51
3	3.6	12.6	7.9	0.0	54
4	3.6	15.6	8.9	1.6	50
5	2.3	12.5	7.8	0.0	45
6	3.8	13.0	8.4	0.0	50
7	1.0	22.3	10.8	0.0	48
8	2.6	19.4	11.7	0.0	56
9	7.6	20.3	14.9	0.0	34
10	7.8	11.3	8.2	0.0	66
11	-1.0	12.8	7.1	0.0	41
12	4.9	17.1	11.3	0.4	56
13	7.1	18.6	10.2	2.4	36
14	-0.1	16.3	8.6	0.0	39
15	5.3	23.8	14.8	0.0	45
16	15.0	25.9	20.3	0.0	38
17	16.1	24.1	20.2	1.4	43
18	9.4	15.5	11.8	0.2	55
19	4.1	18.6	11.8	0.0	47
20	3.7	13.4	9.5	0.6	52
21	5.0	16.0	11.1	0.0	57
22	4.7	23.7	13.9	0.0	22
23	8.6	23.0	15.0	1.2	84
24	7.8	14.3	10.2	0.0	86
25	5.4	15.4	10.4	0.0	49
26	6.1	14.8	11.0	0.0	62
27	10.4	19.4	14.8	0.0	57
28	12.1	21.5	13.5	0.0	38
29	6.4	13.6	9.1	0.0	55
30	-1.1	16.5	7.9	0.0	52
31	4.6	15.2	11.3	0.0	67

Weather data courtesy New Zealand Institute for Horticulture and Food Research, Lincoln research station.

APPENDIX C

BACTERIAL POPULATION DATA FROM ORCHARD SURVEYS (1989, 1990)

Record of Analyses

```
selist
function(xl)
{
# S (or Splus) function:
# xl is a list of vectors.  For each vector,
# the mean (stored in av), the s.e. (stored in se),
# and the number (stored in num), are calculated.
        n <- length(xl)
        av <- array(, n)
        num <- array(, n)
        se <- array(, n)
        for(i in 1:n) {
                xx <- log(xl[[i]])/log(10)
                av[i] <- mean(xx)
                num[i] <- length(xx)
                sd <- sqrt(var(xx))
                se[i] <- sd/sqrt(length(xx))
        }
        xlist <- list(av = av, se = se, num = num)
        xlist
}
```

ORCHARD B (CODE PH)

ph dates

```
[1] 6 10 12 13 14 16 17 19 20 21 23 31 43
```

Total bacterial populations, sample dates 1 - 13 (pht)

```
[[1]]:
 [1]  2.0   1.0  30.0  10.0   1.5  20.0 200.0   1.5  34.0   9.0   5.5   1.5
[13]   1.5   1.0

[[2]]:
 [1]  12.0   2.0 320.0  19.0  37.0   9.0   9.0   1.3   2.0   2.0  16.0   1.0
[13]   7.0   3.0

[[3]]:
 [1]  26.0  20.0  50.0   2.5   3.5  80.0  28.0   5.0   3.0   8.5   2.0   1.0
[13] 125.0   2.0

[[4]]:
 [1]  10  11   4  41  50   2  90   1 120  12   2   1   2   4
```

```
[[5]]:
 [1] 210.0   0.5 160.0  45.0 120.0   3.5  30.0   7.0   0.5  11.5   1.0   2.0
[13]  40.0   1.0   2.5  75.0

[[6]]:
 [1]  275.0   10.0  120.0    1.5 1100.0    1.5   18.0    2.0    0.5    5.5
[11]   60.0    0.5

[[7]]:
 [1]  20.5   5.0 205.0   2.5  65.0   0.5  40.5  45.0  25.0 105.0   1.5   4.5
[13]  14.0   1.5   7.5

[[8]]:
 [1]  15.0  20.0  55.0  15.0  45.0   1.5  65.0  15.0  15.0 335.0  60.0  70.0
[13] 150.0

[[9]]:
 [1]  45.0   1.5  31.0   0.5  10.0  15.0  15.0 280.0 545.0 155.0 215.0  23.0
[13] 210.0 200.0  12.0

[[10]]:
 [1]  85.0  25.0   7.5  12.5   5.0  55.0   1.5 110.0   4.5 140.0 480.0 150.0
[13]   3.5   4.5  65.5   9.0

[[11]]:
 [1]  950   75  375   25   16 1700  380   75 1000  870   50  110   25  270  850
[16]  270

[[12]]:
 [1] 3800 1200  160 2700  640 3800  300  800  900 1100 3200 2000 3100 3000

[[13]]:
 [1] 1500  110 1500  740  450  340  590 2200  990  140  820  100  700 1400 1800
[16] 2400
```

Statistical analysis total bacteria Orchard A (phtstats)

```
$av:
     [,1]     [,2]     [,3]     [,4]     [,5]     [,6]     [,7]     [,8]
0.7364757 0.848796 0.9651221 0.8980687 0.9849843 1.03207 1.062579 1.516335

    [,9]    [,10]    [,11]    [,12]    [,13]
1.54282 1.348787 2.286509 3.13078 2.827691

$se:
     [,1]      [,2]      [,3]      [,4]      [,5]      [,6]      [,7]
0.1903517 0.1807531 0.1795837 0.1902021 0.2292531 0.3181494 0.1961231

     [,8]      [,9]     [,10]     [,11]     [,12]     [,13]
0.1619584 0.2242609 0.184198 0.16359 0.1147779 0.1122526

$num:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12] [,13]
  14   14   14   14   16   12   15   13   15    16    16    14    16
```

E. herbicola populations (phe):

[[1]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[2]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[3]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[4]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[5]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 38.4

[[6]]:
0 0 0 0 0 0 0 0 0 0 0 0

[[7]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[8]]:
0 0 0 0 0 0 0 0 0 0 0 0 143

[[9]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 2.25

[[10]]:
0 0 0 0 0 0 0 0 0 0 0 92.8 92.8 92.8 46.4 139.2

[[11]]:
0 0 0 0 0 0 0 0 0 0 25.1 25.1 25.1 25.1 25.1 50.2

[[12]]:
110 210 31 130 66 210 25 95 140

[[13]]:
0 0 0 0 0 0 0 0 0 0 0 0 112

Average populations E. herbicola Orchard A (pheav):

[1] 0.00 0.00 0.00 0.00 0.00 0.00 0.00 11.00 0.15 29.00
[11] 11.00 110.00 7.00

fl dates

[1] 6 10 11 12 13 14 16 17 19 20 23 31 43

Total bacterial populations, sample dates 1 - 13 (code flt)

[[1]]:
[1] 7.0 20.0 0.5 18.0 36.0 0.5 1.0 3.5 1.5 6.5 2.0 100.0
[13] 0.5 0.5 8.5 30.0 19.0 100.0

[[2]]:
[1] 55 84 7 85 1 44 1 12 600 13 980 13 12 90

[[3]]:
[1] 60.0 5.5 30.0 1.0 25.0 62.0 2.0 5.0 8.5 44.0 16.0 2.0 4.5 5.0 10.0
[16] 90.0 55.0 25.0 50.0

[[4]]:
[1] 135.0 7.5 37.0 0.5 36.5 11.0 3.5 0.5 9.0 11.5 4.5 15.0
[13] 20.0 10.0 5.0 5.0 500.0 35.0 95.0 25.0

[[5]]:
[1] 31 100 37 19 10 130 70 2 100 1 19 34 24 6 830 20 50 17

[[6]]:
[1] 330.0 9.0 110.0 20.0 65.0 9.5 8.5 1.5 5.5 20.0
[11] 360.0 10.0 10.0 1.0 0.5 25.0 20.0 100.0 1040.0 1000.0

[[7]]:
[1] 235.0 23.5 10.0 55.0 3.5 0.5 18.0 3.5 80.0 20.0 105.0 55.0
[13] 185.0 9.5 10.0 200.0 45.0 125.0

[[8]]:
[1] 40.0 375.0 50.0 525.0 290.0 35.0 3.5 12.5 430.0 165.0 10.0 11.0
[13] 45.0 1.5 15.0 8.5 30.0 390.0 250.0 25.0

[[9]]:
[1] 185.0 115.0 105.0 85.0 315.0 145.0 335.0 10.0 350.0 220.0 230.0 110.0
[13] 415.0 310.0 15.0 135.0 17.0 525.0 21.5

[[10]]:
[1] 790 310 1140 675 90 485 350 50 730 560 155 570 165 385 40
[16] 10 15 10 60 305

[[11]]:
[1] 1000 1000 1800 1200 1500 1300 1400 435 295 1370 1230 935 1380 845 600
[16] 1570 1500 1100 700

[[12]]:
[1] 1670 3960 2660 3400 2600 1300 2700 3700 2100 1500 3600 3400 930 3700 310
[16] 4800

[[13]]:
[1] 3400 450 2200 520 1600 2900 540 1300 1500 2300 420 380 270 1900 1700
[16] 3000 180 1100 280 230

Statistical analysis total bacteria Orchard B (fltstats):

$av:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9]
0.7373877 1.44232 1.12672 1.11406 1.428695 1.3862 1.432589 1.646694 2.076405

[,10] [,11] [,12] [,13]
2.227974 3.00946 3.35004 2.951003

$se:
[,1] [,2] [,3] [,4] [,5] [,6] [,7]
0.1873376 0.2348849 0.1349317 0.1621626 0.1580796 0.2079689 0.1695516

[,8] [,9] [,10] [,11] [,12] [,13]
0.1681211 0.1201989 0.1459558 0.04663251 0.0757444 0.09353069

$num:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12] [,13]
18 14 19 20 18 20 18 20 19 20 19 16 20

E. herbicola populations Orchard B (fle)

[[1]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[2]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[3]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[4]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 5

[[5]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[6]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[7]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 15

[[8]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1.5 2.0

[[9]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 10

[[10]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 53 106 106 212 212

[[11]]:
0 0 0 0 0 0 0 0 0 0 0 1350 980 110 270 1360 1050 180 30 40

[[12]]:
0 0 0 0 0 0 0 0 0 720 0.6 0.6 342 310 480 146 0.6

[[13]]:
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 13.6 13.6

Average populations E. herbicola Orchard B (fleav):

[1]0.000 0.000 0.000 0.250 0.000 0.000 0.750 0.188 0.666
[10] 43.000 220.000 126.000 1.700

ORCHARD C (CODE CH)

ch dates

10 12 13 15 16 18 19 20 21 22 23 30 38

Total bacterial populations, sample dates 1 - 13 (cht)

[[1]]:
[1] 0.00 0.10 0.04 0.06 0.02 0.08 0.12 0.30 0.08 0.26 0.38 0.08 0.06 0.06 0.24
[16] 0.44 0.28

[[2]]:
[1] 0.02 0.08 0.06 0.04 4.76 0.16 0.02 0.10 0.12 0.12 0.00 0.10 0.06 0.06 0.04
[16] 0.26 0.04 0.02

[[3]]:
[1] 0.24 0.06 0.68 0.16 6.00 0.48 0.08 5.04 0.06 0.68 0.16 0.04 0.30 0.08 0.32
[16] 0.24 3.76

[[4]]:
[1] 0.72 0.30 0.02 0.02 0.46 0.32 0.12 0.50 0.02 0.20 0.08 0.00 0.08 0.10 0.46
[16] 1.96 0.08 0.14 0.04

[[5]]:
[1] 0.42 0.08 0.04 1.60 0.12 0.16 0.10 0.40 0.10 0.30 0.04 0.70 0.18 0.06 1.64
[16] 0.02 0.22 0.14 0.32 0.16

[[6]]:
[1] 0.06 0.02 0.08 0.48 0.04 0.02 0.04 1.18 0.00 0.06 0.12 38.00
[13] 0.00 0.06 0.32

[[7]]:
[1] 0.02 2.24 0.02 80.00 0.16 14.00 54.00 2.08 0.24 6.00 0.00 0.00
[13] 0.00 0.00 2.00 2.00 16.00 0.00 2.00 6.00

[[8]]:
[1] 0.00 360.00 0.00 74.00 0.04 0.06 152.00 1500.00 1500.00
[10] 270.00 24.00 2.00 62.00 12.00 0.00 24.00 46.00 26.00
[19] 0.00 0.00

[[9]]:
[1] 0.88 6.24 79.80 3.60 79.80 0.02 78.40 0.22 0.56 0.24 0.54 0.02
[13] 0.04 0.00 10.00 1.60 0.00 0.00 0.08 0.02

[[10]]:
[1] 6.00 54.00 18.00 0.46 6.00 8.00 10.00 22.00 0.34

```
[[11]]:
 [1]  4.24 150.00  1.10  0.62 150.00  76.00  4.34  2.66  76.00  0.24
[11]  0.06  2.42  0.60  1.14  0.92

[[12]]:
 [1] 220 160 240 140  60 3500  20 6800  40 6500  46 2400  56 1600   0
[16]   0   0 580 160  20

[[13]]:
 [1] 400 980 360  60 100 100 480 700 1960 220  14  60 240 260   0
[16]   0 420 120 1960
```

Statistical analysis total bacteria Orchard C (chtstats):

```
$av:
      [,1]      [,2]      [,3]      [,4]      [,5]      [,6]       [,7]
-0.7185191 -0.8600341 -0.1979403 -0.6149661 -0.4545061 -0.7417099 -0.05923616

      [,8]      [,9]     [,10]     [,11]     [,12]     [,13]
 0.8148671 -0.1036311 1.076999 0.8329202 2.016249 2.206338

$se:
      [,1]      [,2]      [,3]      [,4]      [,5]      [,6]      [,7]
 0.1214152 0.1442163 0.1633165 0.148913 0.1128072 0.2520259 0.3315101

      [,8]      [,9]     [,10]     [,11]     [,12]     [,13]
 0.4546857 0.3170087 0.2449406 0.2708497 0.4247894 0.3613499

$num:
 [,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12] [,13]
   17   18   17   19   20   15   20   20   20     9    15    20    19
```

E. herbicola populations Orchard C (che):

```
[[1]]:
 [1] 0 0 0 0 0 0 0 0 0 0

[[2]]:
 [1] 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[3]]:
 [1] 2.88 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00

[[4]]:
 [1] 0.04 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[5]]:
 [1] 0.06 2.42 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[6]]:
 [1] 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

[[7]]:
 [1] 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
```

[[8]]:
 [1] 0.06 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[9]]:
 [1] 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[10]]:
 [1] 0.02 18.00 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00

[[11]]:
 [1] 7.00 2.30 7.04 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00

[[12]]:
 [1] 40 60 4800 140 640 360 20 40 0 0 0 0 0 0 0
[16] 0 0 0 0 0

[[13]]:
 [1] 140 100 2000 40 20 0 0 0 0 0 0 0 0 0 0
[16] 0 0 0 0 0

Statistical analysis E. herbicola Orchard C (chestats):

$av:
 [,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9]
  -2 -2 -1.837622 -1.954846 -1.811799 -2 -2 -1.946041 -1.969897

  [,10] [,11] [,12] [,13]
-1.523958 -1.362697 -0.2059304 -0.9072301

$se:
 [,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]
  0 2.093457e-16 0.1623778 0.0451545 0.1420214 0 1.018811e-16 0.05395906

  [,9] [,10] [,11] [,12] [,13]
0.030103 0.3512846 0.317522 0.515423 0.4412458

$num:
 [,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12] [,13]
  10 18 17 20 20 16 19 20 20 10 15 20 20

ORCHARD D (CODE HZ)

hz dates
9 11 13 15 17 19 20 21 22 23 30 38

Total bacterial populations, sample dates 1 - 12 (hzt):

[[1]]:
 [1] 4.96 0.68 0.12 0.44 0.18 0.42 1.00 0.38 0.04 0.18 0.22 0.12 1.70 0.14 0.12
[16] 0.16

[[2]]:
 [1] 0.12 0.14 1.00 0.06 0.06 0.08 0.12 0.06 0.40 0.08 7.32 0.12 0.12 0.12 0.18
[16] 0.14 0.18 0.08 0.02 0.26 1.00

[[3]]:
[1] 2.80 1.04 1.26 1.56 1.14 4.96 0.38 0.78 1.02 2.28 0.20 1.76 2.40 0.24 1.36

[[4]]:
[1] 0.08 0.36 0.08 0.16 0.12 0.58 0.14 0.20 0.12 0.30 0.22 0.22 0.04 0.20 0.12
[16] 0.00 0.04 0.64 0.34 0.16

[[5]]:
[1] 2.40 1.00 0.10 1.70 0.30 5.60 0.44 0.80 0.44 1.78 0.34 0.90 5.30 2.78 4.64
[16] 3.68 0.40 2.68 0.84

[[6]]:
[1] 0.06 0.18 0.00 0.22 1.00 0.26 1.00 0.02 11.00 0.50 0.68 0.46
[13] 0.38 0.26 0.60 0.46 1.88 0.52

[[7]]:
[1] 384 0 0 0 110 0 28 80 40 40 20 10 16 2 56 44 0

[[8]]:
[1] 3.18 3.40 15.00 6.00 12.00 96.00 180.00 26.00 14.00 8.00
[11] 180.00 4.00 1.40 0.04 4.02 5.16 44.00 0.76 20.00 20.00

[[9]]:
[1] 70.00 4.00 72.00 180.00 102.00 16.00 16.00 36.00 100.00 54.00
[11] 26.00 40.00 5.70 38.00 98.00 24.00 50.00 0.36

[[10]]:
[1] 2.8 8.2 3.0 10.0 3.6 6.7 12.0 3.0 23.0 45.0

[[11]]:
[1] 1200 120 480 95 55 1100 0 210 160 160 85 300 570 65 70

[[12]]:
[1] 12000 1900 180 2400 0 4100 200 500 300 60 800 20
[13] 240 0 100 0 1200 0 360 600

Statistical analysis total bacteria Orchard D (hztstats):

> $av:
[,1] [,2] [,3] [,4] [,5] [,6] [,7]
-0.220986 -0.4882555 0.3536294 -0.5528401 0.3598335 -0.1738435 0.7053101

[,8] [,9] [,10] [,11] [,12]
1.241919 1.759069 1.180201 2.290959 1.983796

$se:
[,1] [,2] [,3] [,4] [,5] [,6] [,7]
0.1299458 0.1189982 0.1001149 0.1050397 0.1127369 0.1724379 0.4512268

[,8] [,9] [,10] [,11] [,12]
0.1886765 0.1510713 0.1293055 0.3261234 0.4781997

$num:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12]
16 21 15 20 19 18 17 20 18 10 15 20

E. herbicola populations Orchard D (hze):

```
[[1]]:
 [1] 0.05 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[2]]:
 [1] 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[3]]:
 [1] 0.02 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00

[[4]]:
 [1] 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[5]]:
 [1] 0.04 0.04 0.02 0.02 0.16 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00 0.00 0.00

[[6]]:
 [1] 0.10 0.06 0.02 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
[16] 0.00 0.00 0.00

[[7]]:
 [1] 1600    8   12    6    2    2    0    0    0    0    0    0    0    0    0
[16]    0    0    0    0    0

[[8]]:
 [1] 84.00  0.06  0.02  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
[13]  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00

[[9]]:
 [1] 72.00 24.00  1.36 26.00 28.00 34.00 10.00  0.04  0.36 80.00 80.00  0.00
[13]  0.00  0.00  0.00  0.00  0.00  0.00  0.00

[[10]]:
 [1] 0.00 0.34 0.88 0.36 0.06 1.40 0.26 0.00 0.00 1.70

[[11]]:
 [1]  50 120  35 190  50 160  65  70  25  35   0   0   0   0   0   0


[[12]]:
 [1] 1000  160   20  800  100  100   40   40   60    0    0    0    0    0    0
[16]    0    0    0    0    0
```

Statistical analysis E. herbicola Orchard D (hzestats):

```
> $av:
  [,1]      [,2]      [,3]      [,4]      [,5]      [,6]       [,7]      [,8]
-1.95 -1.969897 -1.919725 -1.969897 -1.774228 -1.834318 -0.9813609 -1.704672

       [,9]      [,10]     [,11]       [,12]
-0.1031281 -0.6290824 0.5696489 -0.04501094
```

$se:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]
0.05 0.030103 0.05469796 0.030103 0.09736134 0.09437929 0.3779069 0.2154309

[,9] [,10] [,11] [,12]
0.4239308 0.3259553 0.5171007 0.5029414

$num:
[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12]
20 20 15 20 20 18 20 20 19 10 16 20

BACTERIAL COUNTS FROM APPLE BLOSSOM SAMPLES IN ORCHARD SURVIVAL TRIALS OF APPLIED EH1087 (1991)

Bacterial numbers from dilution plate counts are given below. Dilution factors are given in brackets below each column of figures. M indicates a missing value.

Orchard Survival Eh1087: Early Blossom

Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
520	11	0	M
372	680	0	0
1360	39	14	0
56	114	28	107
576	94	34	42
836	154	40	0
776	40	0	72
800	708	10	0
500	538	3	146
608	1700	40	120
(5000)	(500)	(5000)	(500)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
0	0	1	M
0	0	3	0
0	0	0	1
0	0	0	0
0	0	0	0
0	0	0	23
0	0	0	29
0	3	0	4
0	1	0	0
0	1	0	M
(undil)	(undil)	(undil)	(undil)

Total Rif^{R} *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
3	0	0	17
0	0	1	0
0	0	0	0
0	0	0	0
0	0	0	0
1	0	0	0
0	0	0	0
2	0	0	0
2	0	0	0
2	0	0	0
(5)	(5)	(5)	(5)

Orchard Survival Eh1087: Mid-blossom

Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
76	66	46	152
73	18	19	52
119	81	86	55
17	154	44	184
17	86	62	184
172	76	31	106
184	15	28	284
67	56	29	184
170	63	0	256
66	33	35	M
(50000)	(500)	(5000)	(5000)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
0	0	1	0
0	0	4	0
6	570	6	120
1	0	0	2
5	0	4	0
13	0	0	1072
60	0	1	30
1	0	1	116
2	0	0	400
(undil)	(500)	(50)	(undil)

Total Rif^{R} *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
1	4	1	12
1	7	2	3
1	16	5	23
3	1	48	68
20	0	0	64
0	0	0	0
0	0	0	0
0	M	0	M
0	M	0	M
0	M	0	M
(5)	(5)	(5)	(5)

Applied Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
14	0	5	0
35	0	4	432
18	2	3	4
35	2	6	2
44	0	7	260
80	3	5	640
34	1	0	89
20	0	13	12
8	1	5	7
12	0	M	17
(50000)	(50000)	(500)	(50)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
7	0	29	28
0	8	3	328
11	28	20	252
17	7	21	136
13	9	25	4
6	0	15	6
35	94	16	41
91	3	21	344
73	5	M	73
95	M	M	560
(500)	(500)	(500)	(50)

Total Rif^R *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
2	3	0	50
12	2	0	33
5	8	7	30
7	1	5	12
7	1	3	13
M	1	3	4
M	8	6	15
M	0	0	27
M	0	0	50
M	M	M	1
(5)	(2500)	(2500)	(250)

Data Manipulations

Means of $\log_{10}$(B x diln factor x bcf + 1) for each sample day were calculated, where B = bacterial count and bcf = blossom correction factor (20). This converts the plate count from 0.1 ml blossom wash to cfu.blossom^{-1}. Plotted values were the back-transformed means of the $\log_{10}$ data.

BACTERIAL COUNTS FROM APPLE BLOSSOM SAMPLES IN GLASSHOUSE SURVIVAL TRIALS OF APPLIED EH1087 (1991)

Bacterial numbers from dilution plate counts are given below. Dilution factors are given in brackets below each column of figures. M indicates a missing value.

Glasshouse Survival Eh1087: Early blossom

Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
1296	3700	1250	134
1000	4250	2500	117
496	2950	584	101
592	2650	1328	46
424	2850	880	416
344	2500	1120	41
732	3150	784	168
764	3600	1152	192
3750	M	152	M
2600	M	16	M
(5000)	(500)	(5000)	(50000)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
1	160	30	70
2	96	512	180
0	148	328	56
0	8	216	M
0	140	440	M
0	32	368	M
0	184	120	M
0	11	720	M
M	188	M	M
M	204	M	M
(100)	(500)	(1000)	(5000)

Total Rif^{R} *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
6	5	2	14
6	5	3	8
10	8	14	11
4	5	21	M
7	2	23	M
2	10	27	M
6	9	24	M
3	0	0	M
M	0	0	M
M	0	0	M
(5)	(5000)	(5000)	(5000)

Glasshouse Survival Eh1087: Mid-blossom

Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
0	220	400	432
180	336	400	288
128	184	280	268
100	240	232	M
164	396	208	M
212	268	256	M
132	218	128	M
100	143	164	M
214	M	136	M
(50000)	(50000)	(50000)	(50000)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
14	47	59	M
2	9	136	M
5	212	288	M
0	54	92	M
0	36	53	M
5	272	7	M
0	152	110	M
1	40	9	M
M	17	11	M
(50)	(5000)	(5000)	

Total Rif^{R} *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
11	8	3	M
0	38	20	M
0	12	26	M
1	10	12	M
52	33	15	M
30	26	6	M
3	10	5	M
3	4	7	M
0	5	16	M
40	M	0	M
(5)	(25000)	(25000)	

Eh1087

DAY 0	DAY 2	DAY 4	DAY 10
14	0	5	0
35	0	4	432
18	2	3	4
35	2	6	2
44	0	7	260
80	3	5	640
34	1	0	89
20	0	13	12
8	1	5	7
12	0	M	17
(50000)	(50000)	(50000)	(50000)

Total *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
2200	42	712	552
2538	72	44	300
864	144	200	608
864	38	504	488
2538	516	16	372
864	87	100	500
864	212	104	480
2538	154	88	448
2538	2	256	M
2538	M	368	M
(undil)	(5000)	(5000)	(5000)

Total Rif^{R} *Erwinia herbicola*

DAY 0	DAY 2	DAY 4	DAY 10
2	23	60	36
2	9	17	46
3	0	13	38
1	25	18	30
0	28	4	36
M	26	60	26
M	9	14	34
M	13	60	34
M	M	7	M
M	M	28	M
(2500)	(25000)	(25000)	(25000)

Data manipulations

Means of $\log_{10}$(B x diln factor x bcf + 1) for each sample day were calculated, where B = bacterial count and bcf = blossom correction factor (20). This converts the plate count from 0.1 ml blossom wash to cfu.blossom^{-1}. Plotted values were the back-transformed means of the $\log_{10}$ data.